

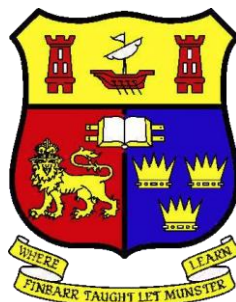
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The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them.

- Sir William Bragg (1862 - 1942)

Identification and characterization of innate immune receptor substrates of γ -secretase enzyme complex

Submitted to the National University of Ireland, Cork,
in fulfilment of the requirements for the degree of



Doctor of Philosophy

by

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Declaration

This thesis has not been previously submitted, in part or in whole, to this or any other University for any degree and is, unless otherwise stated, the original work of the author.

Signed: _____

Jyoti Chhibber

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Abstract

The γ -secretase protease complexes and associated regulated intramembrane proteolysis play an important role in controlling receptor-mediated intracellular signalling events, which have a central role in Alzheimer's disease, cancer progression and immune surveillance. It has previously been reported that the Interleukin-1 receptor, type 1, (IL-1R1) is a substrate for regulated intramembrane proteolysis, mediated by presenilin (PS)-dependent γ -secretase activity. The aims of this project were twofold. Firstly, to determine the conservation of regulated intramembrane proteolysis as a physiological occurrence amongst other cytokine receptors. In this regard, similar to IL-1R1, we identified the Tumour necrosis factor receptor type 1 (TNFR1) and the Toll like receptor 4 (TLR4) as novel γ -secretase substrates. Secondly, given that the diversity of signalling events mediated by the IL-1R1, TLR4 and TNFR1 are spatially segregated, we investigated the spatial distribution, subcellular trafficking and subcellular occurrence of regulated intramembrane proteolysis of IL-1R1, TLR4 and TNFR1. Using dynasore an inhibitor of clathrin-dependent receptor endocytosis, both ectodomain shedding and γ -secretase-mediated cleavage of IL-1R1 were observed post-internalization. In contrast, TNFR-1 underwent ectodomain shedding at the cell surface followed by endosomal γ -secretase-mediated cleavage. Furthermore, immortalised fibroblasts from PS1-deficient mice showed impaired γ -secretase-mediated cleavage of IL-1R1 and TNFR1, indicating that both are cleaved by PS1-and not PS2-containing γ -secretase complexes. Subcellular fractionation and immunofluorescence studies revealed that the γ -secretase generated IL-1R1 ICD translocates to the nucleus on IL-1 β stimulation. These observations further demonstrate the novel PS-dependent means of modulating IL-1 β , LPS and TNF α -mediated immune responses by regulating IL-1R1/TLR4/TNFR1 protein levels within the cells.

Abbreviations

AD	Alzheimer's disease
ADAM	A disintegrin and metalloprotease
AICD	APP intracellular domain
Aph	Anterior pharynx defective
APP	Amyloid precursor protein
ApoE	Apolipoprotein E
A β	Amyloid β
BACE	β -secretase converting enzyme
CCL	CC-chemokine ligand
cDKO	Conditional knock-out
CRD	Cysteine rich domain
CSF-1	Colony-stimulating factor-1
CTF	C-terminal fragment
CXCL	CXC-chemokine ligand 8
DAP	DYIGS and peptidase
DD	Death domain
DR	Death receptor
DISC	Death inducing signalling complex
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase

FAD	Familial Alzheimer disease
FADD	Fas-Associated protein with Death Domain
GSK-3 β	Glycogen synthase-3- β
GnT-V	β 1,6 N-acetylglucosaminyltransferase V
GPCR	G-protein coupled receptor
GSK-3 β	Glycogen synthase kinase-3 β
HMW	High molecular weight
ICAM	Intercellular Adhesion Molecule 1
ICD	Intracellular domain
I-CLiPs	Intramembrane-cleaving proteases
ID	Intermediate domain
IKK	I κ B Kinase
IFN	Interferon
IL-1	Interleukin-1
IL-1R	Interleukin-1 receptor
IL-1Ra	Interleukin-1 receptor antagonist
IL-1RAcP	Interleukin-1 receptor accessory protein
IRAK	Interleukin-1 receptor-associated kinase
JNK	c-Jun N-terminal Kinase
KD	Kinase domain
LAR	Leukocyte-common antigen-related
LPS	Lipopolysachharide

LRP	Lipoprotein-related protein
LT- α	Lymphotoxin- α
MAPK	Mitogen-activated protein kinase
MEKK	Mitogen-activated protein kinase kinase3
MMP	Matrix metalloproteinase
Nav β 2	Voltage-gated sodium channel β 2
NCT	Nicastrin
NFT	Neurofibrillary tangles
NF- κ B	Nuclear factor κ -beta
NTF	N-terminal fragment
PARL	PSEN associated rhomboid-like membrane protease
PBMC	Peripheral blood mononuclear cells
p75 NTR	p75 neurotrophin receptor
Pen	PS enhancer
PI3K	Phosphoinositide-3 kinase
PLAD	Pre-ligand binding assembly domain
PKA	Protein kinase A
PKC	Protein kinase C
PS	Presenilin
Psen	Presenilin
PTM	Post translational modifications
RIP	Regulated intramembrane proteolysis

RIP1	Receptor interacting protein
SDP	Soluble neutrophil-derived serine proteases
SLE	Systemic lupus erythematosus
SP	Senile plaques
SPP	Signal peptide peptidase
TAB	TAK binding protein
TAK	Transforming growth factor β -activated protein kinase
TCR	T-cell receptor
TGN	Trans- <i>golgi</i> network
TLN	Telencephalin
TLR4	Toll like receptor-4
TNF	Tumor necrosis factor
TNF-R	Tumor necrosis factor receptor
TNFSR	Tumor necrosis factor superfamily receptor
TRADD	TNF receptor 1-associated protein
TRAF	TNFR-associated factor
TRID	TNFR1 internalization domain

CHAPTER 1

INTRODUCTION

1.1 Alzheimer's disease and Regulated Intramembrane Proteolysis

1.1.1 Alzheimer's disease

Alzheimer's disease (AD) is a slow progressing, heterogeneous neurodegenerative disorder. On November 3, 1906, Dr. Alois Alzheimer, for the first time discussed the pathology and clinical symptoms of AD, which at the time was termed presenile dementia (Alzheimer et al., 1995). Since its discovery, several proposals have been put forward, in which researchers have speculated that the primary pathologies of AD may be the causative events in the onset of this disease (Armstrong, 2013). The two principal proposals are the amyloid cascade and the Tau hypothesis. Although the primary cause of AD has been the subject of much debate, the amyloid cascade hypothesis has dominated the field of AD research (Piplikar, 2009; Selkoe, 1991). The amyloid cascade hypothesis proposes that the deposition of amyloid- β ($A\beta$) leads to the formation of senile plaques (SPs) and neurofibrillary tangles (NFTs), which in turn are responsible for neuronal cell death, and ultimately dementia (Hardy et al., 1992). However, this hypothesis has been refuted since deposition of $A\beta$ in senile plaques has been reported to be a late event which occurs as a result of other more important pathological changes which have been divided into "positive" changes including lesions such as amyloid plaques, NFTs, glial responses, and "negative" lesions such as neuronal and synaptic loss (Lee et al., 2004; Nalivaeva et al., 2013; Serrano-Pozo et al., 2011). Additionally, $A\beta$ accumulation and deposition has also been shown to induce hyperphosphorylation of tau (Chesser et al., 2013). The tau protein is important for neurite outgrowth and microtubule stabilization. Furthermore, redistribution of tau from the axon to the soma and dendrites followed by its phosphorylation, and deposition within the NFTs has been shown to trigger the AD pathology (Morris et al., 2011). Following these observations, a new "dual pathway" model of causality has been proposed, linking $A\beta$ and tau by separate mechanisms driven by a common

upstream driver including Apolipoprotein E (*ApoE*) mediated increase in plaque and NFTs load, mutations within the Presenilin (*Psen1*) and Presenilin (*Psen2*) and others like glycogen synthase kinase-3 β (GSK-3 β)-mediated tau phosphorylation etc. (Ramachandran et al., 2013; Small et al., 2008).

To date four loci have been associated with early-onset AD; amyloid precursor protein (*APP*), *Psen1*, *Psen2* and *ApoE* (Bekris et al., 2010). The first gene to be identified was the *APP* gene on chromosome number 21 (Goate et al., 1991). The *Psen1* and *Psen2* genes associated with the early-onset AD encode highly homologous integral membrane proteins with eight putative transmembrane domains and till date over 150 *Psen1* and 40 *Psen2* mutations have been identified (Bart De Strooper et al., 2012). These are discussed with relevance to the PS-mediated γ -secretase dependent and independent activity in the later sections. Furthermore, the E4 allele of the *ApoE* on chromosome 19 was identified as a genetic risk factor for both early-onset and late-onset AD and was found to be involved in increased A β deposition and shown to promote A β fibril formation *in vitro* (Bekris et al., 2010).

APP is a type I transmembrane protein which was found to undergo sequential proteolytic cleavage by combination of different enzymes in two different pathways: the non-amyloidogenic or the amyloidogenic pathway (Hui Zheng et al., 2006) **Figure 1**. Irrespective of the pathway involved, the removal of the extracellular domain of APP was found to be a pre-requisite for subsequent cleavage events (Brown et al., 2000). In the non-amyloidogenic pathway or α -pathway, APP is cleaved between lysine-16 and leucine-17 of the A β domain by α -secretase, a member of the “a disintegrin and metalloprotease” (ADAM) family generating a soluble extracellular domain: APP- α (sAPP α) and the membrane bound C-terminal fragment (CTF) 83 (C83) (Anderson et al., 1991). While in the case of amyloidogenic pathway, APP is first cleaved by the aspartyl protease, a β -secretase enzyme (BACE1), releasing the extracellular domain-sAPP β , which is a shorter version of the sAPP α fragment and generating CTF APP 99

(C99) (Kang et al., 1987; Sinha et al., 1999; Vassar, 1999; Yan et al., 1999). The APP CTFs C99 and C83 are then further processed by a transmembrane enzyme complex, known as γ -secretase (Hass et al., 1993). While the γ -secretase-mediated cleavage of C83 gives rise to the hydrophobic peptide p3 and the cytosolic APP intracellular domain (AICD), the C99 cleavage by γ -secretase results in the generation of A β peptides and AICD.

The aberrant accumulation or defective clearance of A β peptides is hypothesized to be critical in the pathogenesis AD (Hardy et al., 1992). These series of sequential proteolytic cleavage events of APP by ADAMs, or BACE and γ -secretase are termed regulated intramembrane proteolysis (Brown et al., 2000).

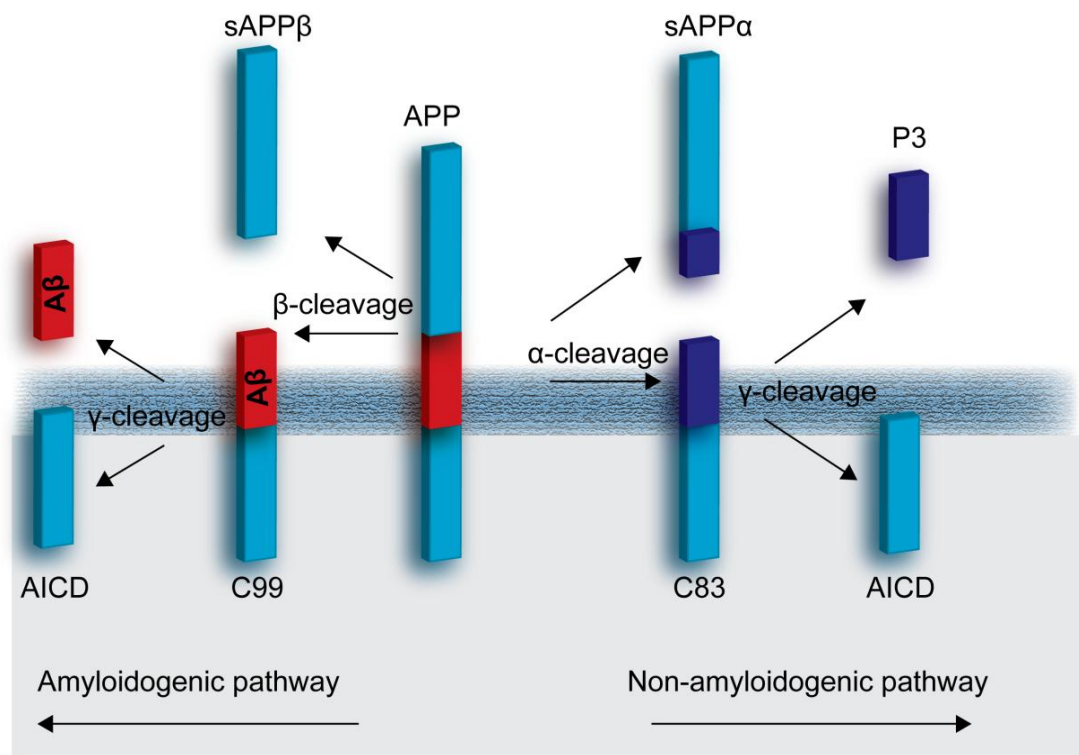


Figure 1. Proteolytic processing of the amyloid precursor protein (APP).

APP is processed by either of the two pathways: the non-amyloidogenic or the amyloidogenic pathway. The right panel shows the non-amyloidogenic pathway, where an initial cleavage by α-secretase generates sAPPα and C83. On the left panel, in amyloidogenic pathway APP is first cleaved by β-secretase to generate sAPPβ and C99. C83 and C99 from both pathways are subsequently processed by the γ-secretase enzyme complex. Proteolysis by γ-secretase at the ε-site generates the APP intracellular domain (AICD) and either p3 or Aβ peptides (Adapted from Cole & Vassar, 2007).

1.1.2 Regulated Intramembrane Proteolysis

The conventional view of receptor-mediated cell signalling holds that plasma membrane receptors upon binding of their ligand relay signals across membranes, and intracellular enzymes or changes in second messengers facilitate the propagation of ligand-receptor initiated signal. Following ligand binding, most receptors either undergo ectodomain shedding or internalization, thereby negatively regulating or terminating the ligand-receptor generated signal. Once internalized a receptor can be either degraded or recycled back to the plasma membrane. Additionally, in a few cases, like G-protein coupled receptors (GPCR), binding of an inhibitory protein acts to terminate the signalling (Madhus et al., 2009).

Recently, a new signalling pathway has emerged, whereby cell surface receptor signalling events are regulated by sequential proteolysis. Regulated intramembrane proteolysis influences a diverse range of processes such as lipid metabolism, cellular differentiation, gene transactivation and the response to unfolded proteins (Hass et al., 2009). The functional significance of regulated intramembrane proteolysis, particularly γ -secretase-mediated processing, has gained much attention lately owing to its involvement in many vital processes in the cell and in some diseases. Regulated intramembrane proteolysis describes a novel regulated proteolytic cleavage event in which specific receptors that first undergo ectodomain shedding, are subsequently cleaved within the lipid bilayer to liberate an intracellular domain (ICD) (Brown et al., 2000). Regulated intramembrane proteolysis is a two step process. First, multiple proteases in a highly regulated proteolytic cleavage event at the cell surface, cleave transmembrane proteins forming soluble extracellular domain (Lichtenthaler et al., 2007; Weber et al., 2012). These proteases collectively termed sheddases belong to one of the following families of proteases: ADAMs (Edwards et al., 2008), matrix metalloproteinases (MMP) (Hinsbergh et al., 2008); and soluble neurotrophil-derived

serine proteases (SDPs) (Lin et al., 2008). Following ectodomain shedding, the membrane-bound carboxyl terminal fragment (CTF) is cleaved by Intramembrane-cleaving proteases (I-CLiPs), which results in the release of the membrane bound or soluble ICD **Figure 2**. It has also been suggested that the main function of iCLiP family members was to regulate degradation of integral membrane proteins, but with time the released fragments have acquired signalling properties allowing regulated signalling by some substrates (Kopan et al., 2004).

I-CLiPs are multispinning, integral membrane proteins containing classic protease motifs within their transmembrane domains (Wolfe, 2009). I-CLiPs family members include S2P metalloproteases, rhomboid serine proteases and GxGD aspartyl proteases which consist of γ -secretase and signal peptide peptidase (SPP) **Table 1.1**. The substrates for the I-CLiP members undergo a prior cleavage in the extracellular domain, except rhomboid proteases which have the ability to process full length substrates with large ectodomain (Strisovsky, 2013). The S2P catalytic activity is dependent on its conserved HEXXH motif, which contributes two histidines and one glutamate, and a distally located conserved aspartate residue found within the LDG motif (Wolfe, 2009). SPP and γ -secretase protease complex share the highly conserved GxGD and YD motifs containing the two critical catalytic aspartate residues necessary for their enzymatic activity and located within the transmembrane domain (Tomita et al., 2013). Rhomboids on the other hand contain transmembrane serine, histidine, and asparagine catalytic triad and a serine-histidine dyad (Strisovsky, 2013). Among the iCLiP family members, γ -secretase has been most widely studied owing to its role in neuropathogenesis of AD.

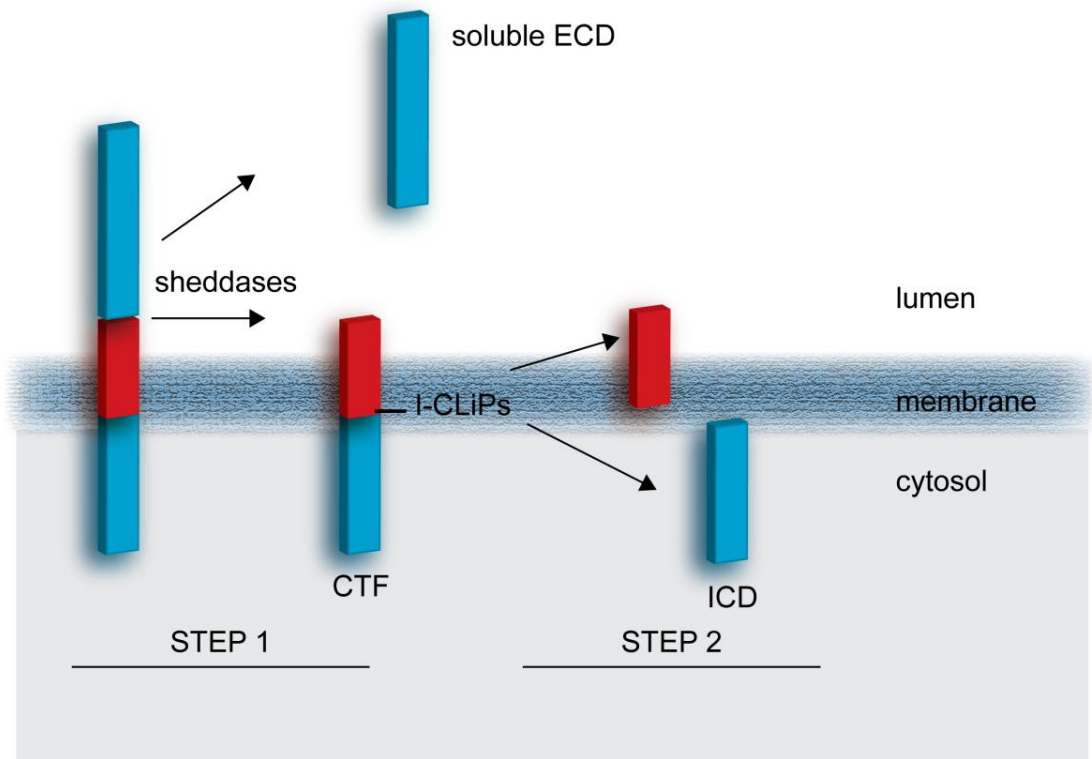


Figure 2. Regulated Intramembrane Proteolysis involves the sequential proteolytic cleavage of select transmembrane receptors

Step 1: An initial cut at a site outside the membrane by sheddases such as ADAMs, MMPs and SDPs shortens the protein to less than 30 amino acids in the extracellular domain, generating a soluble extracellular domain and CTF. Step 2: The second cut is performed on CTF by I-CLiP family members within the transmembrane domain releasing the ICD portion of the protein (Adapted from Wakabayashi & Strooper, 2008).

Table 1.1 The iCLiP family members and their substrates
(R. Kopan et al., 2004)

iCLiP family	Substrate topology	Substrate
Aspartyl proteases		
γ -secretase Signal-peptide peptidase	Type I Type II	See table 1.4 MHC-class-I signal peptides
Metalloproteases		
Site-2 protease Eep	Type II Type II	SREBP, ATF6 cAD1 pheromone precursor
Serine proteases		
Rhomboid Aar A	Type I Type I	Spitz, Keren and Gurken Unknownl

Type I topology- amino terminus is extracellular, carboxyl terminus is cytoplasmic
Type II topology- amino terminus is cytoplasmic, carboxyl terminus is extracellular
ATF6- Activating transcription factor-6
EGFR- Epidermal growth factor receptor
MHC- Major histocompatibility complex
PSH-Presenilin homologue
SREBP- Sterol regulatory element binding protein

1.2 The γ -secretase protease complexes: The protein quartet

Christian Hass and Denis J. Selkoe gave the name γ -secretase to a proteolytic enzyme which, in combination with BACE1 cleaved APP resulting in the generation of A β peptides associated with AD pathologies (Christian Hass et al., 1993). In 1995, Peter St George Hyslop and co-workers identified pathogenic mutations in the encoding gene *Psen1* in several families with early-onset AD, which subsequently led to the discovery of the multipass membrane proteins-PS1 and PS2 (Sherrington et al., 1995; Tanzi et al., 1996). Mutations in the homologous *Psen1* and *Psen2* genes were found to cause the most common and aggressive form of familial AD (FAD). In an attempt to purify the PS-dependent γ -secretase activity, PS proteins (PS1 and PS2) were consistently pulled down as part of a high molecular weight multi-protein complex. Later, with the help of high-grade biochemical purification studies and genetic screens, γ -secretase was discovered to be an oligomeric complex consisting of at least four integral membrane proteins: PS, nicastrin (NCT), anterior pharynx defective-1 or-2 (Aph-1/Aph-2) and PS enhancer-2 (Pen-2) (Francis et al., 2002; Goutte et al., 2002; Strooper, 2003a) **Figure 3**. Further the genetic ablation or RNAi knockdown of either of the components, and reconstitution of these four elements in *S. cerevisiae*, a cell type devoid of intrinsic γ -secretase, demonstrated that these components were sufficient for complete γ -secretase activity (Kaether et al., 2006).

1.2.1 Presenilin proteins and γ -secretase protease

Structurally, PS proteins are polytopic transmembrane domain proteins with nine membrane-spanning regions (transmembrane domains 1-9) (Laudon et al., 2005; Steiner et al., 2008) located in the ER, the intermediate compartment, the *cis*-Golgi region and plasma membrane (Annaert et al., 1999; Culvenor et al., 1997; Kim et al., 2000; Ray et al., 1999; Walter et al., 1996). Following translation, the endoproteolysis of the PS holoproteins is triggered by the assembly of active γ -secretase complexes which generate stable, non-covalently associated N-terminal fragment/C-terminal fragment (NTF/CTF) heterodimers (Dries et al., 2008; Podlisny et al., 1997; Thinakaran et al., 1996; Ward et al., 1996). An unidentified protease named presenilinase is known to mediate the endoproteolytic cleavage of PS proteins. It has been suggested that presenilinase is either PS or γ -secretase, since catalytic defunct PS mutants cannot be endoproteolysed (Xia et al., 2008).

A wealth of biochemical evidence supports the theory that PS proteins facilitate γ -secretase activity. Firstly, mutations in either of the two PS homologues (PS1 or PS2) alter A β production, and genetic ablation of PS1 dramatically impairs γ -secretase cleavage of APP for example, PS1 Δ 9, T291-S319del, PS1-L166P, PS1-G384A, PS1-A246E and PS2 N141I (Bentahir et al., 2006); secondly, the types of compounds that inhibit γ -secretase contain moieties typically found in aspartyl protease inhibitors; thirdly, studies aimed at purifying γ -secretase concluded that involvement of the PS proteins in γ -secretase activity requires incorporation of the PS-NTF/CTF heterodimer into HMW-protein complexes (250-1000-kDa in size), which contain at least three additional PS-binding molecules: NCT, Aph-1/2 and Pen-1/2 (Marjaux & Hartmann, 2004; Gu et al., 2003; Strooper, 2003b). It is the assembly of these functionally conserved proteins that results in the formation of catalytically active PS-dependent γ -secretase complexes (Shirotani et al., 2004) **Figure 4.**

1.2.2 NCT

NCT is a type I transmembrane glycoprotein. After PS proteins, NCT was the next component of γ -secretase complex to be detected via PS1 immunoprecipitation in conjunction with mass spectrometry (Dries et al., 2008). The N-terminal region of NCT transmembrane domain interacts with the C-terminus of PS, facilitating the assembly of NCT into γ -secretase complex (Capell et al., 2003). The interaction of NCT with substrates is considered to be critical for them to be cleaved and for this reason NCT has always been considered as the “gatekeeper” of γ -secretase complex (Wakabayashi et al., 2008). The extracellular domain of NCT was reported to be a docking site for the incoming γ -secretase substrate (Shah et al., 2005). This interaction was found to be mediated by the carboxylate side chain of a glutamate residue (E333) present in the DYIGS and peptidase (DAP) domain of NCT, the site of binding to α -amino group of the available N-terminus of ectodomain-shed substrates (Dries et al., 2008). However, it was indicated that E333 was essential for the maturation of γ -secretase but dispensable for its catalytic activity (Chávez-Gutiérrez et al., 2008).

1.2.3 Aph-1

A seven transmembrane domain-containing protein, Aph-1, was identified as γ -secretase complex member through the discovery of an Aph-1 deficiency in *C.elegans* (Yu et al., 2000). This deficiency was found to exhibit a similar phenotype to the PS (sel-12 and hop-1 in *C. elegans*) and NCT (*aph-2* in *C. elegans*) mutants of the same species (Francis et al., 2002). Further to this, Aph-1 mutant embryos were found to be aberrant in NCT trafficking as seen in PS defective embryos (Francis et al., 2002) and the absence of Aph-1 was associated with decreased processing of PS proteins (Takasugi et al., 2003). Further studies demonstrated that Aph-1 interacts and stabilizes the immature form of NCT (pre-complex) in the ER or *cis*-Golgi which then binds to the PS holoprotein (Dries et al., 2008; Gu et al., 2003). Interestingly upon the

stable transfection of cells with Aph-1 no differences in A β secretion were observed. This was later attributed to the role played in maturation of γ -secretase complex by another limiting factor-Pen-2 (Seong Hun Kim et al., 2003).

1.2.4 Pen-2

Pen-2 was identified using a genetic screen in *C. elegans* for loci that interact with Notch, PS and NCT (Francis et al., 2002). Furthermore, the incorporation of Pen-2 into γ -secretase complex was shown to trigger endoproteolysis of the PS holoprotein into its stable heterodimer (Dries et al., 2008). Binding of Pen-2 is mediated through the fourth transmembrane domain of PS proteins and this event subsequently prompts the final steps of the γ -secretase maturation process with the concomitant final glycosylation of NCT and release of the γ -secretase enzyme complex from ER to the Golgi (Capell et al., 2005) **Figure 4.**

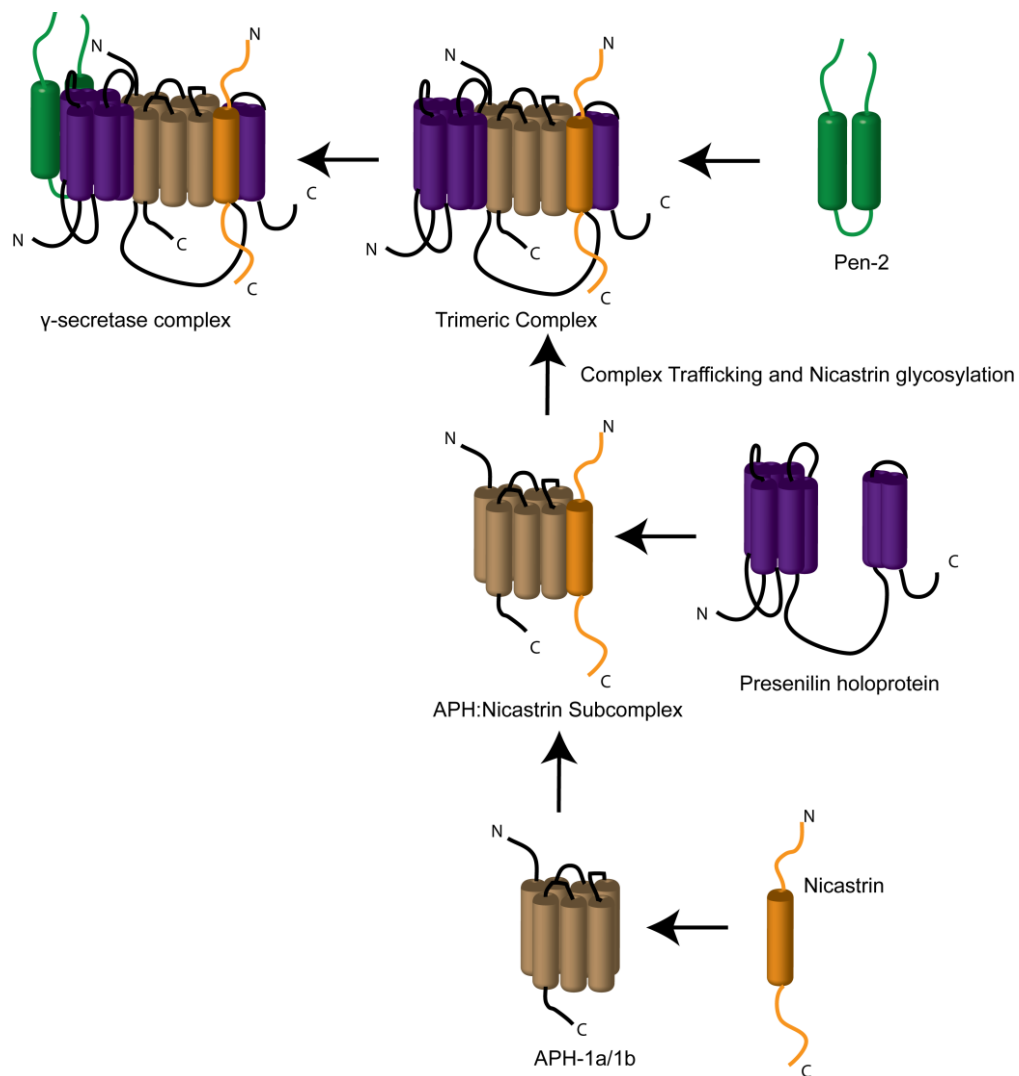


Figure 4 A schematic representation of the formation of γ -secretase complex.

Evidence shows that PS, NCT, Aph-1, and Pen-2 are the only components of γ -secretase complex. NCT and Aph-1 are involved in the early formation of the complex, interacting with each other and forming a dimeric sub-complex. The PS holoprotein is incorporated into the Aph-1: NCT sub-complex, NCT undergoes post-translational modifications and the complex is transported to the cell surface (or other $A\beta$ generating compartments, i.e. TGN) as a trimeric complex. Pen-2 is incorporated into the complex and PS is cleaved, thus forming the active γ -secretase complex (Adapted from Verdile, Gandy, & Martins, 2007).

1.3 γ -secretase dependent and independent functions of PS proteins

1.3.1 γ -secretase dependent functions of PS proteins

After their discovery as the catalytic components of the γ -secretase enzyme complex responsible for processing APP, PS proteins were a major target for anti-amyloidogenic therapy (Hui Wang et al., 2012). However, PS knock-out mice models which were generated to study and define the essential roles of PS proteins in AD pathology highlighted the severe side effects posed by the therapeutic use of γ -secretase inhibitors (Yan et al., 2010). Additionally, the discovery of over 91 substrates with varied functions pointed out a more general role of PS-dependent γ -secretase-mediated cleavage (Haapasalo et al., 2012). PS/ γ -secretase-mediated cleavage of proteins was shown to affect biological processes in several ways, 1) by removing the membrane bound CTF's responsible for signalling, thus inhibiting key signalling events, 2) processing of proteins by γ -secretase enzyme complex acting to enhance or inhibit its interaction with the cytosolic adaptor proteins, hence directing the output of the signalling event, or 3) the product of the γ -secretase-mediated cleavage in most cases was a transcriptionally active ICD, which regulated gene transcription (Haapasalo et al., 2012). **Table 1.2** lists the known γ -secretase cleavage products which translocate to the nucleus and mediate various functions.

Table 1.2 Type-I γ -secretase substrates and suggested roles of their γ -secretase-mediated cleavage products

Substrate	Product	Function	Reference
Alcadin α/γ	Alc-ICD	Suppression of AICD-Fe65-mediated transcription	Araki Y et al., 2003; Araki Y et al., 2004
APLP1/APLP2	ALID1	Transcription regulation with Fe65 and Tip60	Scheinfeld MH et al., 2002; Walsh DM et al., 2003; Eggert S et al., 2004; Pardossi-Piquard R et al., 2005
A β PP	AICD	Transcription regulation with Fe65 and Tip60; neurodegeneration	May P et al., 2002; Hoe HS et al., 2005
Betacellulin	BTC-ICD	Cell growth inhibition	Stoeck A et al., 2010
CD43	CD43-ICD	CD43 signalling?	Anderson CX et al., 2004; Anderson CX et al., 2005; Mambole A et al., 2008
CD44	CD44-ICD	Transcription regulation, cell transformation, macrophage fusion	Okamoto I et al., 2001; Lammich S et al., 2002; Murakami D et al., 2003; Cui W et al., 2006; Pelletier L et al., 2006
Delta 1	DICD	Transcription regulation, antagonizing of Notch signalling	Chu J et al., 2002; Ikeuchi T et al., 2003; LaVoie et al., 2003; Bland CE et al., 2003; Six E et al., 2003
EpCAM	Ep-ICD	Regulation of β -catenin/Lef-1-mediated transcription; oncogenic	Maetzel et al., 2009
EphA4	EphA4-ICD	Enhancement of dendritic spine	Inoue E et al., 2009
Ephrin B1	eB1ICD	Regulation of ephrinB1 signalling related to process outgrowth, transcriptional regulation	Tomita T et al., 2006
ErbB4	s80 or E4ICD	Apoptosis, regulation of transcription and p53 levels, oligodendrocyte maturation, regulation of astrogensis	Linggi B. et al., 2006; Srinivasan R. et al., 2000; Ni CY. et al., 2001; Lee HJ. et al., 2002; Williams CC. et al., 2004; Vidal GA. et al., 2005; Lia C. et al., 2004; Arasada RR. et al., 2005; Omerovic J. et al., 2004
GHR	GHR-ICD	Transcriptional regulation?	Cowan JW. et al., 2005
IFNaR2	IFNaR2-ICD	Regulation of STAT-mediated transcription	Saleh AZ. et al., 2004; El Fiky A. et al., 2005
Jagged 2	JICD	Antagonizing of Notch signalling; regulation of AP-1-mediated transcription	Chu J. et al., 2002; Ikeuchi T. et al., 2003; LaVoie MJ. et al., 2003
LAR	LICD	Regulation of β -catenin/TCF/LEF-mediated transcription	Haapasalo A. et al., 2007
LRP1 (LDLR)	LRP1-ICD	Transcriptional regulation, negative regulation of AICD-Fe65-Tip60-mediated transcription, ischemic cell death	Hemming ML. et al., 2008; Polavarapu R. et al., 2008; May P. et al., 2002; Kinoshita A. et al., 2003
LRP1 b	LRP1 b-ICD	Transcriptional regulation, tumor suppression	Liu CX. et al., 2007
Na- β 2	β 2-ICD	Regulation of cell adhesion and migration; Regulation of Na function	Kim DY. et al., 2005; Kim DY. et al., 2007
Neuregulin-1	NRG1-ICD	Transcriptional regulation of PSD-95	Bao J. et al., 2003; Bao J. et al., 2004
Notch-1/Notch-2/Notch-3/Notch-4	NICD	Transcriptional regulation (CSL-mediated)	Saxena MT. et al., 2001; Shimizu K. et al., 2000
p75NTR	p75NTR-ICD	Regulation of trk-p75NTR complex formation, transcriptional regulation, nuclear entry of NRIF and apoptosis; potentiation of trk receptor signalling	Kenchappa RS. et al., 2006; Jung KM. et al., 2003; Kanning KC. et al., 2003; Zampieri N. et al., 2005; Domeniconi M. et al., 2005; Ceni C. et al., 2010
RAGE	RICD	Apoptosis	Galichet A. et al., 2008
RPTPk	RPTPk-PIC	Activation of β -catenin/TCF/LEF-mediated transcription opposite to the full length RPTPk	Anders L. et al., 2006
SorLA	SorLA-ICD	Transcriptional regulation	Hermey G. et al., 2006; Nyborg AC. et al., 2006; Bohm C. et al., 2006
Syndecan-3	SICD	Transcriptional regulation, regulation of CASK subcellular localization	Schulz JG. et al., 2003

As highlighted in **Table 1.2**, PS/ γ -secretase is involved in the proteolytic processing of a diversity of receptors with varied functions. This diversity emphasized the need to generate PS knock-out mouse models to further study the effect of loss of PS/ γ -secretase. In the attempt to create these mouse models, deletion of *Psen1* was shown to be embryonically lethal (Donoviel et al., 1999), while in contrast *Psen2* deficiency was associated with no major pathologies, apart from an age-associated mild pulmonary phenotype, but *Psen2*-deficiency enhanced the embryonic lethal phenotype of *Psen1* deficiency (Herreman et al., 1999). A role for PS2 in A β production

has also been highlighted, where loss of PS2 activity has revealed significant deficits in proteolytic processing of APP indicating a defect in γ -secretase activity (Steiner et al., 1999; Toda et al., 2011). In this context it was interesting to note that the PS1^{+/-}PS2^{+/-} mice remained healthy, clearly indicating a critical minimum threshold, below which the drop of *Psen* gene dosage and function result in serious risks (Tournoy et al., 2004). In the following years, this limitation which was hindering further studies aimed at determining the functions of PS proteins was overcome by the development of tissue-specific *Psen*-knock-out models. Selective disruption of *Psen1* in the mouse forebrain during post-natal life enabled the tissue-specific deletion of *Psen1* (Feng et al., 2002; Guo & Fu et al., 1999; Wang et al., 2004; Xia et al., 2001). In addition, the selective knock-out of one or more alleles of *Psen1* genes, with the retention of at least one functional copy was used (Tournoy et al., 2004). Additionally, both PS1 and PS2 FAD mutant transgenic lines were generated using heterologous promoters exhibiting transgene expression patterns varying from neuron-specific to ubiquitous (Wang et al., 2004; Wen et al., 2004, 2002). In this regard the PS1^{+/-}PS2^{-/-} 'partial deficient' mice had the most severe reduction in PS alleles, still permitting post-natal survival allowing the analysis of long-term partial inhibition of γ -secretase *in vivo* (Donoviel et al., 1999). Furthermore, deficiency in PS proteins in lymphoid T and B cells antagonized T cell homeostasis and signalling (Maraver et al., 2007), while B cells presented with a deficit in both lipopolysaccharide (LPS) and B-cell antigen receptor-induced proliferation and signal transduction events (Yagi et al., 2008). The phenotype of various PS knock-out mice models and transgenic animals studied is listed in **Table 1.3**.

Table 1.3 Phenotypes of presenilin transgenic animals

Genotype	Phenotype	References
PS1 ^{-/-}	Severe developmental abnormalities and perinatal lethality	Shen J. et al., 1997; De Strooper, B. et al., 1998
PS2 ^{-/-}	No abnormal pathology but develop mild pulmonary fibrosis and haemorrhage with age	Winker, E. et al., 2009
PS1 ^{-/-} and PS2 ^{-/-}	Lethal, embryos die late in embryogenesis. Embryos at day 9.5 display severe growth retardation; forebrain degeneration and ventricle enlargement	Herreman, A. et al., 2000; Feng R. et al., 2004
PS1 ^{+/-} PS2 ^{+/-}	Viable and fertile. After 6 months, they develop skin lesions and an autoimmune disease similar to SLE	Tournoy, J. et al., 2004
PS1 ^{+/-} PS2 ^{-/-}	Remain healthy	Tournoy, J. et al., 2004
PS1 brain-specific conditional KO	Reduced A β production and increased inflammatory responses	Beglopoulos V. et al., 2004
Cerebral cortex	APP CTFs differentially accumulate in the cerebral cortex, generation of β -amyloid peptides is reduced; enhanced oxidative stress and astrogliosis	Yu, H. et al., 2001; Feng Gu. et al., 2008; Zhu M. et al., 2008
Excitatory neurons of forebrain	Reduced Clearance of Hippocampal Memory Traces	Feng, R. et al., 2001,
PS1-FAD knock-in	Differential effects on A β production, amyloid deposition and neuronal vulnerability	Spitzer, P. et al., 2010, Wang, R. et al., 2004
PS1 P117L	Neurogenesis promoted by overexpression of wild type PS1 but not by FAD mutant; FAD mutant impaired survival of NPCs leading to fewer new neurons produced.	Wen et al., 2002; Wen et al., 2004
A246E on PS1 ^{-/-} background	NPC proliferation increased, fractional survival of NPCs less, no net change in new neuron production	Chevallier et al., 2005
M146V KI on PS1 ^{-/-} background	Reduced NPC proliferation by 25% in FAD mutant	Wang et al., 2004
P264L KI	No change in DCX-positive immature neurons	Zhang et al., 2007
M146L or PS1DE9	No difference in NPC proliferation or neurogenesis in FAD mutants	Choi et al., 2008

Interestingly, the study of the various *Psen* KO mouse models and transgenic animals has highlighted the role of PS/ γ -secretase in the immune system. The importance of γ -secretase-mediated proteolysis in neuroinflammatory responses was first highlighted when several groups demonstrated the association of activated microglia and reactive astrocytes with amyloid plaques, along with upregulated expression of cell-surface proteins (MHC class II molecules, CD11b and scavenger receptors), cytokines (tumor-necrosis factor (TNF)), interleukin-6 (IL-6 and IL-1) and chemokines (CXC-chemokine ligand 8 (CXCL8) and CC-chemokine ligand-3 (CCL3)) (Weiner et al., 2006). Interestingly, studies in PS Δ KO mice models led to a new perspective, where at 3 months of age inflammatory changes including small inducible cytokine A27, complement component C1q α - β , cathepsins D and S, CD antigens and MHC histocompatibility proteins were recognized as initial pathological events (Beglopoulos et al., 2004; Dong et al., 2007),

which were followed by activated microglia and reactive astrocytes associated with amyloid plaques in addition to synaptic loss, tau hyperphosphorylation and subsequent neurodegeneration (Saura et al., 2004). The involvement of PS proteins/ γ -secretase in immune system was further strengthened by *in vivo* *Psen1*^{-/+} *Psen2*^{-/-} ‘partial knock-out’ mouse models which had a severe autoimmune phenotype characterized by glomerulonephritis, keratitis, dermatitis and vasculitis, furthermore, with age these mice developed skin hyperplasia, increased CD4⁺/CD8⁺ T cell ratio and B cell infiltrates in several tissues (Tournoy et al., 2004). In addition to the brain inflammation, FAD-linked PS1 M146L and PS1 M5 transgenic mice models showed defective T-cell activation and higher susceptibility of lymphocytes to cell death compared to their wild-type counterparts (Eckert et al., 2001). Further studies led to the hypothesis that PS/ γ -secretase-dependent Notch signalling positively influenced selection and development of T cells by modifying TCR signalling and these mice models being defective in γ -secretase cleavage-mediated Notch signalling demonstrated decreased CD8⁺ T cells (Germain, 2002). Additionally, a role of PS proteins in B cell function was also reported, where loss of PS function resulted in reduced LPS and B cell antigen receptor-induced proliferation and signalling events (Yagi et al., 2008). This has also been credited to processing of CD46 by PS/ γ -secretase in response to pathogens which in turn stimulates CD46-dependent T cell responses (Weyand et al., 2010). Jayadev and co-workers have also reported a γ -secretase-dependent PS2 role in microglia activation by suppressing the proinflammatory responses, with IFN γ up-regulating PS2 expression and PS2 deficiency resulting in enhanced cytokine expression possibly by targeting various immune receptors (Jayadev et al., 2010).

Along with neurodegeneration and immune system a role of PS/ γ -secretase-mediated cleavage on cancer has also been demonstrated. For example, γ -secretase-mediated cleavage of L1 cell adhesion molecule (L1-ICAM) is essential for nuclear signalling of L1

in human carcinoma cells (Riedle et al., 2009), Notch ICD is oncogenic (Takebe et al., 2013), p75NTR ICD has been reported to have a role in glioma invasion (Johnston et al., 2007; Wang et al., 2008), and CD-44 ICD has been shown to promote neoplastic transformation of rat fibroblastic cells (Nagano et al., 2004; Pelletier et al., 2006). Recently a number of γ -secretase-independent role of PS proteins have also emerged discussed in the next section **Figure 5**.

1.3.2 γ -secretase independent functions of PS proteins

Apart from the involvement in γ -secretase-mediated cleavage events, independent research reports have demonstrated that the PS proteins are involved in numerous other cell-signalling pathways independent of their γ -secretase activity (Koo, 2004). Using yeast-two-hybrid or co-immunoprecipitation approaches the PS proteins have been shown to interact with over 30 proteins (Chen et al., 2002; McCarthy et al., 2009). In some cases, while the biological significance of the association with the PS proteins is not apparently obvious, these associated proteins can be subdivided into functional groups, including those involved in γ -secretase protein complex, Wnt signalling, cell-cell adhesion, vesicular transport, calcium signalling and apoptosis (Ilagan, 2004; Koo, 2004; Parent, 2004).

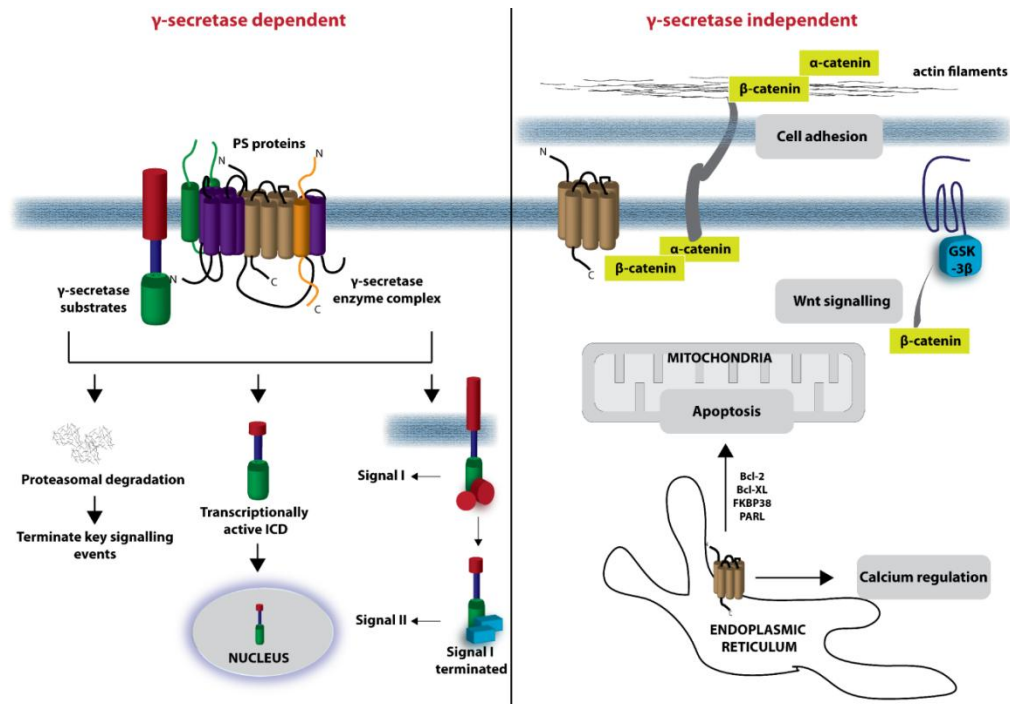


Figure 5 Presenilin: γ-secretase dependent and independent functions

The left panel shows the role played by PS proteins in RIP of proteins and subsequent signalling events. The right panel highlights the emerging functions of PS proteins in various pathways-cell adhesion, Wnt signalling, apoptosis and calcium regulation (Adapted from Hass et al., 2009).

1.3.2.1 PS proteins and β -catenin

Among the PS interacting proteins, β -catenin has been most widely studied. The membrane bound β -catenin interacts with cadherin family members like E (epithelial) and N (neural) cadherin, which are γ -secretase substrates linking them to α -catenin and enabling the formation of a linkage between adherence junctions and the cytoskeleton (Parisiadou et al., 2004). In contrast as a cytoplasmic protein, β -catenin translocates to the nucleus to initiate canonical Wnt signalling (Killick et al., 2001). Studies have linked β -catenin with PS proteins, where β -catenin interacts with the cytoplasmic domain of PS1, which then targets β -catenin for protein kinase A (PKA) and GSK-3 β -mediated degradation, requiring the formation of a scaffold complex comprising of Axin and APC (Cox et al., 2000; Huang and Xi He, 2009; Murayama et al., 1998, 1999; Noll et al., 2000). Furthermore, the expression of wild-type PS1 as well as PS1 with FAD-linked mutations stabilizes the E-cadherin/ β -catenin/ α -catenin complex demonstrating that it is γ -secretase independent function of PS1 (Baki et al., 2001). PS1 has also been shown to function in phosphoinositide-3 kinase/ Akt (PI3K) signalling pathway and regulate the association of PI3K regulatory subunit p85 with N-or E-cadherin, resulting in inactivation of GSK3- β , thereby preventing GSK3- β mediated degradation of β -catenin (Baki et al., 2008a). The hypothesis that PS1/ β -catenin/cadherin interactions are PS dependent but γ -secretase independent has been challenged by the impaired γ -secretase activity and exacerbated amyloid pathology seen in PS1 hydrophilic loop deleted mice models (Zhu et al., 2011a) and the emerging evidence suggesting that PS1 regulates β -catenin-mediated cell adhesion and signalling through γ -secretase-mediated cleavage of leukocyte-common antigen-related (LAR) receptor tyrosine phosphatase (Haapasalo et al., 2007).

1.3.2.2 PS proteins and calcium signalling

PS proteins have also been implicated in calcium signalling (Wu et al., 2013). Evidence shows that PS proteins localize predominantly in the ER and have been suggested to function as ER Ca^{2+} leak channels (Area-Gomez et al., 2009; J. Walter et al., 1996). This is supported by the studies with fibroblasts from FAD patients containing PS1-A246E mutation (Ito et al., 1994), cultured neural cells expressing PS1 L286V (Guo et al., 1996; Guo et al., 1997), *Xenopus* oocytes injected with cRNA encoding PS1-M146V and PS2-N141I FAD mutants (Leissring et al., 1999), synaptosomes and cortical neurons from PS1-M146V mutant mice (Guo et al., 1997; Stutzmann et al., 2004) and in hippocampal neurons from PS2-N141I transgenic mice (Guo and Sebastian, et al., 1999; Schneider et al., 2001). Additionally, the γ -secretase independent interaction between PS and proteins involved in calcium signalling, including IP_3 receptor, ryanodine receptor, sarco-/endoplasmic reticulum Ca^{2+} ATPase (SERCA) further strengthens this hypothesis (Zhang et al., 2013). On the contrary, the observation that a large proportion of AD family members show altered Ca^{2+} release prior to the development of AD, have led to studies which depict the defect in ER Ca^{2+} signalling is caused by the many FAD-linked PS mutations (Das et al., 2012; Etcheberrigaray et al., 1998). Thus suggesting that the long-term Ca^{2+} signalling dysregulation is the early diagnostic change occurring long before histopathological features for AD, which in turn further increase Ca^{2+} levels, leading to a feed-forward loop. Supporting this view, increased Ca^{2+} levels have been functionally linked to the major features and risk factors of AD: ApoE4 expression, PS and APP mutations, amyloid β plaques, apoptosis, hyperphosphorylation of tau and synaptic dysfunction (Stutzmann, 2007).

1.3.2.3 PS proteins and apoptosis

The role of PS proteins in apoptosis was highlighted when FAD-linked PS variants, or the conditional deletion of *Psen1/Psen2* in adult mouse brains led to progressive loss of synapses, dendrites, and neurons, suggesting that loss of *Psen* function, caused neuronal degeneration which was credited to increased apoptosis (Saura et al., 2004). This was further established when the expression of PS2 CTF rescued mouse T-cell hybridomas from T-cell receptor and Fas-induced apoptosis (Vito et al., 1996), additionally, conditional deletion of *Psen1/Psen2* in thymocytes increased their resistance to anti-CD3-induced apoptosis *in vivo* (Maraver et al., 2007) and PS1-deficient neurons with increased caspase-3 activation and apoptosis, were rescued by expression of wild-type PS1 but not by FAD-linked PS1 mutants (Baki et al., 2008b) while the primary hippocampal neurons from PS1 M146V knock-in mice displayed increased apoptosis and also increased neuronal vulnerability to focal ischemia as compared to their wild-type controls (Guo and Sebastian, et al., 1999; Mattson et al., 2000). On the contrary a γ -secretase independent function of PS proteins in apoptosis was highlighted when the primary cortical neurons from *Psen1* P264L knock-in mice did not exhibit increased degeneration, (Siman et al., 2001, 2000).

PS proteins have also been suggested to alter apoptosis directly through their interactions with mitochondrial associated proteins like Bcl-2 and Bcl-XL (Alberici, 1999; Passer, 1999), the two major anti-apoptotic members of the Bcl-2 family and other apoptosis related proteins such as FKBP38 (an immunophilin family member residing in the mitochondrial membrane), Omi/HtrA2 and PARL (PSEN-associated rhomboid-like inner mitochondrial membrane protease) (Gupta et al., 2004; Pellegrini et al., 2001; Wang et al., 2005). The pro-apoptotic effect of *Psen2* over-expression is associated with down-regulation of Bcl-2, whereas the anti-apoptotic effect of *Psen* deficiency in thymocytes is linked to the elevation of Bcl-XL expression (Maraver et al., 2007). A more recent study shows that apoptotic stimulation of cells leads to caspase-

mediated cleavage of PS proteins (Kim, 1997; Walter et al., 1998), where inhibition of caspase-mediated cleavage of PS1 in cells makes them more susceptible to apoptosis, while caspases-mediated cleavage of PS2 generates the Met298-Ile448 intracellular PS2 fragment with an anti-apoptotic function (Vito, 1997). Furthermore, as previously discussed an indirect effect of PS proteins on apoptosis has also been suggested owing to their role in both calcium signalling and A β generation.

1.3.2.4 PS proteins and protein trafficking

Another γ -secretase-independent PS function which has been reported is its role in protein trafficking. PS proteins interact with a number of vesicle transport proteins like syntaxins, or Rab family members' (Dumanchin et al., 1999; Kametani et al., 2004; Scheper et al., 2004; Smith et al., 2000; Suga et al., 2004). Additionally, PS1 is known to regulate the glycosylation and intracellular trafficking of components of γ -secretase complex (Wang et al., 2004). This is supported by the studies showing that the loss of PS1 function leads to increased maturation and cell surface accumulation of A β PP, a γ -secretase substrate (Cai et al., 2003) and also non γ -secretase substrates like transferrin, again highlighting a γ -secretase independent function of PS proteins in protein trafficking (Zhang et al., 2006). PS proteins also function at the lysosomal levels to degrade proteins through the autophagosome–lysosome system, where PS1 interacts with and affects the trafficking of telencephalin (TLN or ICAM-5) to affect autophagic vacuole maturation (Neely et al., 2011). Loss of PS1 function leads to abnormal accumulation of TLN in autophagic vacuoles and is rescued by over-expression of either the wild type PS1 or γ -secretase inactive PS1 D257A mutant and is not mimicked by the use of γ -secretase inhibitors, thus highlighting that it is a γ -secretase independent function of PS1 (Esselens et al., 2004). Furthermore, PS1-deficient neurons accumulate degradative organelles containing α and β -synuclein consistent with a physiological role for PS1 in the regulation of neuronal organelle trafficking (Wilson et al., 2004). As a result, it has also been proposed that PS proteins

are chaperone/trafficking proteins, which bring the substrate and protease together and/or assemble γ -secretase complex.

1.4 The many substrates of γ -secretase

Since the identification of APP, 91 new γ -secretase substrates have been identified using proteomic, genetic and biochemical methods (Hemming et al., 2008), with distinct functions like regulating cell fate, adhesion, migration, neurite outgrowth, or synaptogenesis (Haapasalo et al., 2012). Despite the increasing number of reported γ -secretase substrates, there has been no consensus regarding the characteristic features amongst the substrates facilitating their recognition by the γ -secretase enzyme complex. However, from the rigorous characterisation of some known γ -secretase substrates like Notch, APP, IL-1R1 and ErbB4, some common features and regulatory mechanisms have emerged.

Firstly, most known γ -secretase substrates are found to be type-I integral membrane proteins. However, there are exceptions and substrates of other topologies like β 1,6 N-acetylglucosaminyltransferase V (GnT-V) and Glutamate Receptor Subunit 3 have been reported (Haapasalo et al., 2012; Meyer et al., 2003; Nakahara et al., 2006). Secondly, most of these substrates undergo ectodomain shedding, a pre-requisite to γ -secretase-mediated cleavage of the remaining membrane bound CTF (Haapasalo et al., 2012; Selkoe et al., 2007; Yan et al., 2010). The ectodomain shedding is generally mediated by ADAM family members and in most cases is constitutive, but it can also be induced by several stimuli, such as ligand binding, protein kinase C (PKC) activation by phorbol esters, or Ca^{2+} influx (Edwards et al., 2008). Some of the currently characterized γ -secretase substrates, such as lipoprotein-related protein-1 (LRP) or voltage-gated sodium channel β 2 subunit (Nav β 2), have been shown to undergo an alternative N-terminal cleavage by BACE1 in a similar manner to APP β (Haapasalo et al., 2012). Though it is still unclear whether sheddases are responsible for controlling

substrate specificity by reducing ectodomain size. While not all receptors that undergo ectodomain shedding are cleaved by γ -secretase like tumor necrosis factor (TNF) receptor type 2 (TNF-R2) and the transferrin receptor (Hemming et al., 2008), a general pre-requisite for ectodomain shedding is considered to be the defining characteristic of potential γ -secretase substrates. Additionally, the identification of γ -secretase substrates with diverse topologies also makes it difficult to predict consensus structural sequences and the fact that a single substrate is cleaved at multiple sites for example for APP, CD44, Notch1-4 (Kopan et al., 2004) makes it even harder. For example, mutagenesis studies of PS1 showed that single amino acid substitutions can alter the preference for Notch cleavage over APP. Despite such varied characteristics, there are some common features shared by most of these substrates listed in **Table 1.4**.

Table 1.4 Characteristic features of the known γ -secretase substrates

(Adapted from Annakaisa Haapasalo, 2012)

Characteristic features of known γ -secretase substrates
<ol style="list-style-type: none"> 1. Type-I transmembrane proteins * 2. Lack of the ECD is considered to be the defining feature of a potential γ-secretase substrate ** 3. PS/γ-secretase mediated RIP occurs at or near the transmembrane and cytoplasmic domains 4. Loss of PS/γ-secretase activity results in no ICD formation with a simultaneous CTF accumulation 5. Most ICDs are degraded by proteasome and some translocate to the nucleus 6. Substrate trafficking controls access to protease

Exceptions

* Beta 1,6 N-acetylglucosaminyltransferase V, Glutamate Receptor Subunit 3

** Transferrin, TNF-R2

1.5 Post translational modifications and spatiotemporal regulation of γ -secretase substrates

Apart from the characteristic features of known γ -secretase substrates, it is likely that there are other types of substrate recognition that are required to facilitate γ -secretase-mediated cleavage of the membrane-bound stub, including post-translational modifications (PTMs) and spatiotemporal regulation.

The reversible PTM of a large number of proteins has a ubiquitous role in the regulation of protein functions and activities in a diverse range of cellular functions. It is therefore not surprising that PTMs are important in directly or indirectly modulating the activity of the γ -secretase enzyme complex itself, for example PS proteins undergo proteolysis, phosphorylation and ubiquitination; additionally glycosylation of NCT is also essential for the trafficking of PS proteins (Herreman, 2003). PTMs also affect the processing of γ -secretase substrates. In this context, 1) palmitoylation of p75 neurotrophin receptor (p75NTR)-CTF (Underwood et al., 2008), 2) ubiquitination of the human Notch-CTF (Gupta-Rossi et al., 2004), 3) increased phosphorylation of APP-CTFs (Vingtdeux et al., 2005) and 4) palmitoylation and phosphorylation of CD44-CTFs (Thorne et al., 2004) have been shown to facilitate their processing by γ -secretase. PTMs also tend to regulate γ -secretase-mediated cleavage of substrates in an indirect manner. For example, (1) hypoglycosylation of LRP1 has been reported to facilitate extracellular cleavage resulting in increased production of LRP1 CTF, a substrate precursor for γ -secretase-mediated cleavage (May et al., 2003); (2) palmitoylation of betacellulin is not required for ADAM10-dependent cleavage in its extracellular domain, but is necessary for the stability of betacellulin-CTF to generate betacellulin-ICD (Stoeck et al., 2010). Interestingly, there have been reports where PTMs prevent the substrates from being targeted for γ -secretase-dependent processing as in the case of Mucin 1 (MUC1), where N-glycosylated MUC1 heterodimers are not processed

by sheddases to generate MUC1 CTF thus preventing their cleavage by γ -secretase enzyme complex (Julian et al., 2011).

Apart from regulating stability of γ -secretase substrates or enhancing their processing by the γ -secretase enzyme complex, PTMs also regulate trafficking of substrates and their γ -secretase generated cleavage products between distinct subcellular membrane compartments. For example, 1) palmitoylation of betacellulin is not only required for the synthesis of betacellulin-ICD but is also crucial for its localization to the nuclear-membrane (Stoeck et al., 2010). The importance of PTM in regulating trafficking of substrates is further highlighted by the evidence that regulation of proteolysis can also be co-ordinated at a higher resolution within the plane of the organelle's membrane (Grbovic et al., 2003; Gupta-Rossi et al., 2004; Hass et al., 2009; Urra et al., 2007).

The role of PS proteins in trafficking of γ -secretase substrates and their cleavage products has recently been highlighted, where 1) PS1 deficient cells or cells expressing γ -secretase inactive PS Asp mutants demonstrate increased cell surface accumulation of APP and APP CTFs (Cai et al., 2003; Leem et al., 2002). Additionally, a delay in internalization of APP is seen in PS1 deficient cells, supported by the observation that FAD mutations in PS1 lead to decreased release of APP-containing vesicles and reduce cell surface APP (Kaether et al., 2002; Kim et al., 2005; Leem et al., 2002; Réchards et al., 2006). 2) PS1 deficiency *in vivo* or the use of γ -secretase inhibitors also leads to aberrant accumulation of tyrosinase and its CTFs in post-Golgi vesicles (Wang et al., 2006). One explanation for the role of PS proteins in γ -secretase-dependent protein trafficking is that some γ -secretase substrates mediate protein transport. For example SorLA, Sortilin and SorCS1b are members of the mammalian Vps10p sorting receptor family that bind to various cargo proteins and regulate the sorting and trafficking of a diverse array of protein (Andersen et al., 2005; Böhm et al., 2006; Nyborg et al., 2006). Therefore, some trafficking deficits in PS deficient models may be attributed to altered metabolism of Vps10p proteins.

1.6 Regulated intramembrane proteolysis of immune receptors

Ectodomain shedding is a pre-requisite to γ -secretase-mediated proteolysis. A role for ectodomain shedding in the regulation of cytokine signalling has been elucidated for several receptors, including TNFR1/TNFR2 and IL-1R1/IL-1R2 generating soluble receptor ectodomain. As previously discussed PS/ γ -secretase play an important role in regulating immune system (**Section 1.3.1**). Further to this, a growing list of immune receptors undergoing regulated intramembrane proteolysis highlight the importance of these cleavage events in cytokine signalling **Table 1.5**. Relevant to the current study we will focus on the IL-1, TLR and TNF superfamily members, discussing their role in A β /AD, neuroinflammation and highlighting the current knowledge about their interplay with PS/ γ -secretase.

Table 1.5 List of immune receptors subjected to γ -secretase-mediated cleavage.

Substrate	Function	Function of ICD	Reference
CD43	Lymphocyte surface receptor	Membrane clearance of receptor-derived CTD	Mambole, A. et al., 2008
CD44	Cell-surface glycoprotein	Gene transactivation activity	Lammich, S. et al., 2002
CLA-A2	MHC class I protein	Unknown	Carey, B. W. et al., 2007
CXCL16	Chemokine ligands	Unknown	Schulte, A. et al., 2007
CX3CL1	Chemokine ligands	Unknown	Schulte, A. et al., 2007
IFN γ R2	Subunit of type I IFN γ receptor	Gene transactivation activity	Saleh, A. Z. et al., 2004
IL-1RI	Cytokine receptor	Unknown	Elzinga, B. M. et al., 2009
IL-1RII	Cytokine receptor	Unknown	Kuhn, P. H. et al., 2007
IL6R	Cytokine receptor	Unknown	Chalaris et al., 2010
Notch 1-4	Signalling receptors	Gene transactivation activity	Donoviel et al., 1999

1.6.1 IL-1

The inheritance of certain IL-1 gene variants have been associated with AD (Nicoll et al., 2000). For instance, a polymorphism in the promoter region of *IL-1 α* (-899) or *IL-1 β* (exon 5) has been shown to triple the risk for AD, even a greater risk is associated with a composite *IL-1 α* (-899) and *IL-1 β* exon 5 polymorphism (Grimaldi et al., 2000). In 1989, Griffin and co-workers for the first time highlighted the importance of IL-1 β -mediated signalling in AD pathology, reporting a thirty-fold increase in IL-1 β expression by glial cells associated with amyloid-plaques (Alvarez et al., 1996; Griffin et al., 1989; Sheng et al., 1998), later increased plasma and blood levels of IL-1 β were also seen in patients with AD (Licastro et al., 2000). In an attempt to better understand the mechanism and effect of increased IL-1 β in AD patients, a cell type specific, IL-1 β -mediated positive regulation of the γ -secretase mediated proteolytic processing of APP and the secretion of the A β -peptides (Buxbaum et al., 1992), through a c-Jun N-terminal Kinase (JNK)-dependent mitogen-activated protein kinase (MAPK) pathway was observed (Liao et al., 2004). Furthermore, IL-1 β was also shown to directly impair microglial phagocytosis of A β in *in vitro* cell cultures thus negatively regulating the clearance of A β (Koenigsknecht-Talboo et al., 2005). However, studies carried out in mouse models have suggested that elevated IL-1 levels and resulting neuroinflammatory response might not be completely detrimental in the case of AD (Shaftel et al., 2008). On the contrary, more recent studies have highlighted a neuroprotective role of IL-1 β . For instance, inhibition of IL-1 β signalling in the brain has been shown to be associated with brain atrophy, and modified levels of APP and PS1 (Oprica et al., 2007), further APP^{swe}/PS1^{dE9} transgenic animals over-expressing IL-1 β in the hippocampus show a reduction in A β peptide levels and plaque burden, with no effect on A β processing or APP expression at transcription level (Matousek et al., 2012; Shaftel et al., 2007).

IL-1Ra, sIL-1R1 and sIL-1R2 bind to IL-1 β with a higher affinity than IL-1R1, and thereby negatively modulate the IL-1R1 signalling cascade. In this context, increased levels of sIL-1R2 was also found in the cerebrospinal fluid (Garlind et al., 1999). The observed elevation of sIL-1R2 in AD fitted with the finding that IL-1R2 undergoes α - and β -secretase-mediated ectodomain shedding followed by γ -secretase-mediated cleavage (Kuhn et al., 2007). Additionally, upregulated levels of IL-1Ra was found in peripheral blood mononuclear cells (PBMCs) of AD patients (Mizwicki et al., 2013) while the IL-1Ra knock-out mice exhibited increased microglial activation and neuronal damage induced by intracerebroventricular infusion of human A β peptide (Craft et al., 2005). Additionally, IL-1R1 is the major mediator of IL-1 β -induced signalling. However, an IL-1R1 independent role in AD is indicated by the observation that IL-1R1 knock-out does not affect A β deposition in Tg2576 mice and does not alter efficiency following A β immunotherapy (Das et al., 2006). Activation of protein kinase C (PKC) can increase the processing and secretion of APP (Yang et al., 2007). Similarly, IL-1R1 ectodomain shedding is inducible by PKC activation (Elzinga et al., 2009). Interestingly, our group reported IL-1R1 to undergo similar sequential cleavage first by sheddases followed by γ -secretase enzyme complex. Importantly, we demonstrated that inhibition of γ -secretase activity antagonised IL-1-induced cytokine secretion, suggesting that PS/ γ -secretase regulate IL-1-induced cytokine responsiveness (Elzinga et al., 2009). In addition to IL-1R1 and IL-1R2, IL6R has also been reported as a γ -secretase substrate (Chalaris et al., 2010; Kuhn et al., 2007).

The importance of IL-1-induced cytokine responsiveness in AD and the regulated intramembrane proteolysis of IL-1 receptors is further highlighted by upregulated transcription/secretion of cytokines IL-1, IL-6 and chemokines CCLs and CXCLs by A β (Mizwicki et al., 2013). These observations clearly implicate an important role for IL-1R1 in both AD and other inflammatory diseases.

1.6.2 TLR

Despite contrary results, a role of IL-1 β -induced signalling has been suggested in microglial activation and clearance of A β in AD brain. TLR family members have also been linked with AD and neuroinflammation, where ligands for TLR2, TLR4 and TLR9 show enhanced phagocytic uptake of A β peptides and play a role in the activation of microglial cells (Chen et al., 2006; Iribarren et al., 2005; Jin et al., 2008; Tahara et al., 2008; Trudler et al., 2010). Further to this, A β peptides activate typical TLR signalling pathways via MyD88 and NF- κ B (Jana et al., 2009). This has been validated by the knock down of MyD88 expression in AD mouse model, which shows reduced inflammation and accelerated cognitive deficits, indicating a protective role of TLR-mediated signalling via MyD88 (Michaud et al., 2011), and up-regulation of NF- κ B-regulated microRNA (miRNA-146a) in response to A β (Cui et al., 2010).

Studies in *tlr2*^{-/-} mice have demonstrated that TLR2 is required for activation of microglial cells and the clearance of A β peptides, as *tlr2*^{-/-} mice are unresponsive to A β 42 and show accelerated cognitive decline (Frank et al., 2009; Jana et al., 2009; Richard et al., 2008). In addition to TLR2 a role for TLR4 has been demonstrated, as the administration of an acute injection of the TLR4 ligand, lipopolysaccharide (LPS), in AD transgenic mice models reduces A β plaque burden, suggesting that TLR4 activation also facilitates A β uptake (DiCarlo et al., 2001; Herber et al., 2007). Additionally, spontaneous loss-of-function mutation in the *tlr4* gene strongly inhibits microglial and monocytic activation by aggregated A β resulting in a significantly lower release of the inflammatory products IL-6, TNF- α and nitric oxide (Walter et al., 2007). This has been further supported by research in the AD mouse model (Hu APPswe PS1dE9) homozygous for a destructive mutation of *tlr4* which demonstrates increased levels of diffuse and fibrillar A β deposits as well as soluble and insoluble A β in the brain, along with TLR4-dependent down-regulation of IL-1 β , IL-10, IL-17 and TNF- α cytokines (Jin et al., 2008). The clinical relevance of this observation is highlighted by marked up-

regulation of *tlr4* mRNA in APP transgenic mice and increased expression of TLR4 in AD brain tissue associated with amyloid plaque deposition (Walter et al., 2007). There are also studies correlating TLRs with the PS/ γ -secretase. For instance, a role of TLRs in monocyte/macrophage differentiation by regulating the γ -secretase-mediated regulated intramembrane proteolysis of the colony-stimulating factor 1 (CSF-1) receptor has been demonstrated (Trudler et al., 2010). Additionally, integrative computational analysis of whole-genome datasets has led to the discovery of defective TLR4-mediated signalling in PS-deficient B cells (Yagi et al., 2008). The benefits of TLR4-mediated signalling are further supported by their neuroprotective benefits of TLR4-mediated signalling in stroke (Marsh et al., 2010). The study carried out with the TLR4 agonist called monophosphoryl lipid A (MPL) which significantly reduces A β levels in brain, mitigates AD-related pathology, and improves cognitive function with negligible side effects in the APP^{swe}/PS1 mouse model of AD (Buchanan et al., 2010; Michaud et al., 2013; Wang, 2013). These observations highlight a neuroprotective role of TLR4-mediated signalling in inflammatory etiologies of the nervous system.

1.6.3 TNF

Apart from increased levels of sIL-1R2, a 25-fold increase in TNF α levels was observed in cerebrospinal fluid of AD patients. Following which AD associated region in TNF gene was verified by studies correlating AD and TNF haplotype via sibling pair families (Perry et al., 2001). Interestingly, the genes for TNF receptors, *Tnfr1* (chromosome 1p) and *Tnfr2* (chromosome 12p) also showed genetic linkage to late-onset AD, while only *Tnfr2* (exon6) polymorphism was found to be associated with late-onset AD (Perry et al., 2001). On the contrary, increased expression of TNFR1 in the AD brain was observed (Li et al., 2004). These observations were supported by the studies in PS and AD transgenic mouse models, whereby TNF- α and interferon (IFN)- γ induced A β production (Klegeris et al., 1997), and deletion of either of these cytokine receptors-TNFR1 or IFN- γ receptor type I resulted in decreased A β plaque formation and

inflammation, as well as learning and memory deficits (Yamamoto et al., 2007). TNF α -mediated stimulation of γ -secretase activity by JNK-dependent phosphorylation of PS1 and NCT (Kuo et al., 2008) could be one mechanism via which TNF α induces A β production. Thus the inhibition of inflammatory cytokine TNF- α is seen as a potential approach to AD (Tobinick et al., 2008).

This is further highlighted by the functions mediated by this cytokine and its receptors. For instance the increased generation of TNF α by monocytes and macrophages in AD brain is generally accompanied by the induction of iNOS and shown to mediate neurotoxicity (Combs et al., 2001). Also, A β interacts with TNF α to induce neuronal damage via reactive oxygen species (ROS) and NO-dependent pathways with the involvement of JNK/c-Jun and NF- κ B with the involvement of JNK/c-Jun and NF- κ B (Combs et al., 2001; Eberhardt et al., 1996; Goodwin et al., 1995). The neuronal loss in AD brain has also been attributed to apoptosis. In this context A β binding to TNFR1 has been suggested to induce neuronal apoptosis (Li et al., 2004). Additionally, TNF binding to neuronal Cd120a/b receptors is suggested to trigger caspase activation via increased release of TNF by the microglia and caspase-3 activation (Pickering et al., 2005). Interestingly, the TACE enzyme responsible for cleaving the membrane bound TNF α , TNFR1 and TNFR2 is also upregulated in some neuroinflammatory conditions.

1.7 Interleukin-1 superfamily

The discovery of interleukin-1 (IL-1) cytokines and further insight into their mechanism of action has led to a better understanding of the host-immune response to injuries, infections and several diseases. Since its discovery, the IL-1 superfamily has expanded and as of now consists of 11 cytokines, 13 receptors, including a subgroup of decoy and adaptor receptors (Garlanda et al., 2013; Smith, 2000) These are briefly described in **Figure 6**.

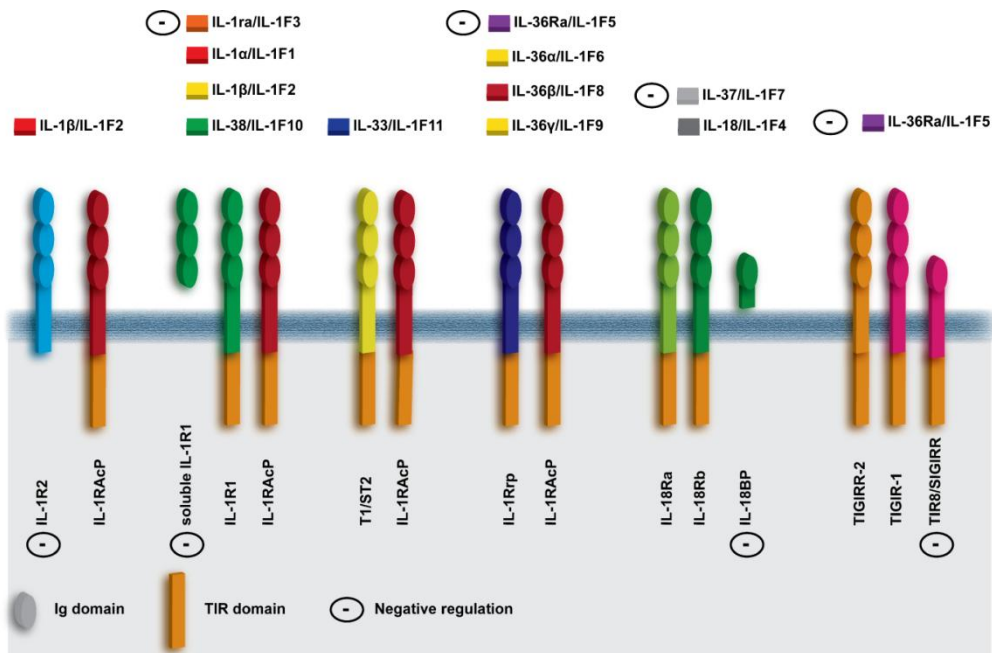


Figure 6 Members of the IL-1 superfamily

The IL-1R family includes the receptors (IL-1R1, IL-18R, T1/ST2, and IL-1Rrp2) and the accessory proteins (IL-1RAcP) for IL-1, IL-18, IL-33, and other IL-1 family members (IL-36a, IL-36b, and IL-36g). IL-1Ra, soluble IL-1R1, IL-36Ra, IL-37, IL-18 binding protein (IL-18BP), IL-1R2, and TIR8/SIGIRR are negative regulators acting at different levels, as receptor antagonists, decoy receptors, scavengers, or dominant negative. Single immunoglobulin IL-1R related receptor (SIGIRR), three Ig IL-1R related molecule-1 and molecule-2 9TIGIRR-1, and TIGIRR-2) are still orphan receptors (Adapted from Riva et al., 2012).

1.7.1 IL-1 cytokines

IL-1 α and IL-1 β are ubiquitously expressed and are synthesized as precursor proteins, pro IL-1 α and pro IL-1 β which are cleaved by calpain and interleukin converting enzyme (ICE) respectively forming the mature IL-1 α and IL-1 β **Figure 7** (Dinarello et al., 1995). Pro IL-1 α and mature IL-1 α are biologically active; whereas pro IL-1 β is biologically inactive and requires cleavage (Dinarello, 2006). A third member of the IL-1 cytokine family which inhibits the binding and biological activities of both IL-1 α and IL-1 β , is called interleukin-1 receptor (IL-1R) antagonist (IL-1Ra) (Arend, 1991). IL-1Ra binds to IL-1R1 with a similar affinity but fails to transduce a signal (Eisenberg et al., 1990). Thus, acting to regulate the IL-1 α / β mediated signalling through the IL-1R1. Studies of mice deficient in IL-1R1, IL-1 α , IL-1 β or double deficient in IL-1 α and IL-1 β , show no particular phenotype and exhibit normal homeostasis, but are distinctly different from IL-1Ra deficient mice (Horai et al., 1998; Zheng et al., 1995). IL-1Ra deficient mice exhibit abnormal reproduction, stunted growth and develop spontaneous diseases such as rheumatoid arthritis (Hirsch et al., 1996). However, in case of externally induced diseases, these mouse models highlight the role played by these three IL-1 members in disease severity. For example- mice lacking IL-1R1 fail to develop proliferative lesions of vascular smooth muscle cells in mechanically injured arteries (Rectenwald et al., 2000). Also IL-1 β deficient mice do not develop destructive joint processes following injection of streptococcal wall components (Van Den Berg, 2001). IL-1 α deficient mice have brisk inflammatory response to turpentine-induced inflammation while the IL-1 β deficient mice have no response (Horai et al., 1998). These differences can be attributed to the fact that IL-1 β has an important role in the positive regulation of immune responses, while IL-1 α acts to down-regulate the effect of IL-1 β (Boraschi et al., 2005). Due to their ability to induce a broad spectrum of systemic changes in neurological, endocrinologic, hematologic and metabolic systems, IL-1 cytokines are seen as potential therapeutic targets for various inflammatory

diseases (Barrie et al., 2005; Navarro-González et al., 2008; Numerof et al., 2005). The strategies used so far include targeting the enzymes responsible for activating IL-1 α and IL-1 β cytokines post cleavage from their precursor proteins (Miller et al., 1993), blocking IL-1 α / β cytokines using the receptor or ligand antagonists and targeted gene disruption (Luheshi et al., 2009). However, the clinical administration of these antagonists, IL-1Ra and antibodies to IL-1 receptors is limited due to the complexity of delivering these proteins, thus reducing their usefulness (Dinarello, 2011). Furthermore, it is critical to develop a better understanding of the signalling pathways regulated or affected by these cytokines before targeting them and developing therapeutics (Akdis et al., 2011; Dinarello, 2011; Luheshi et al., 2009).

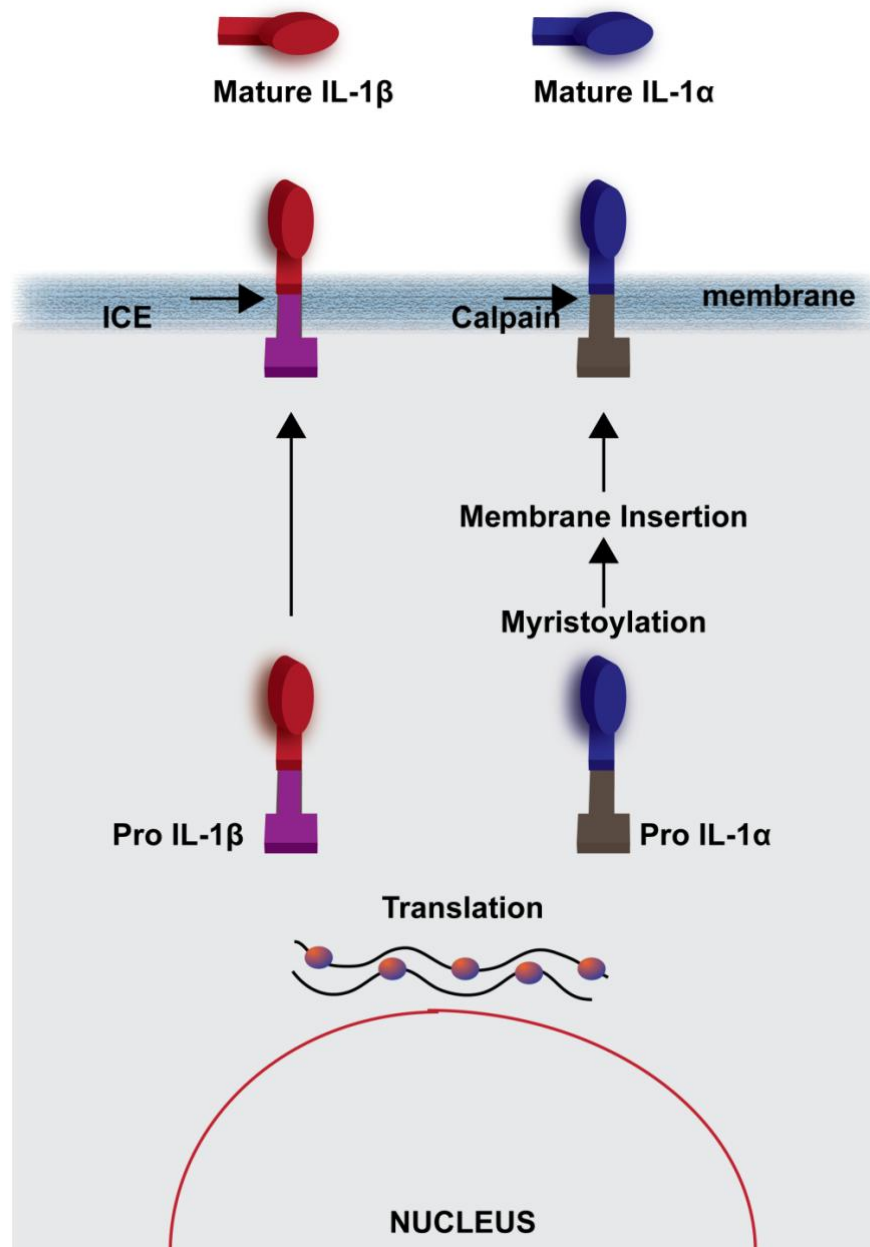


Figure 7 Proteolytic processing of IL-1 α and IL-1 β .

Precursor of IL-1 α and IL-1 β (pro-IL-1 α and IL-1 β) are translated and translocated to the cytosol. These are then transported to the cell surface, where they are cleaved by the intracellular IL-1 β converting enzyme (ICE), generating mature IL-1 α and IL-1 β which are secreted into the extracellular space (Adapted from Dinarello and Margolis, 1995).

1.7.2 IL-1R1

IL-1R1, indispensable for IL-1 α and IL-1 β mediated effects, is a type-1 transmembrane protein with an extracellular N-terminus, an intracellular C-terminus, and a single transmembrane domain (Sims et al., 1988). Studies showed that, antibodies to IL-1R1 blocked IL-1-mediated responses in most cell types (Campbell, 2012; Schäfers et al., 2001; Sommer et al., 1999). Additionally, in IL-1R1-deficient mice, IL-1 responses such as increased IL-6 production, fever, acute-phase response, delayed type hypersensitivity, and the ability to combat infection by *Listeria* were impaired (Glaccum et al., 1997), highlighting its importance in various signalling pathways.

IL-1R1 extracellular fragment consists of three immunoglobulin (Ig) domains. Two of these Ig domains are required for effective binding to the ligand and one is essential for binding to the IL-1R1 accessory protein (IL-1R1 AcP) which is essential for IL-1 mediated signalling and only binds to the IL-1R1-ligand complex (Greenfeder et al., 1995; Lu et al., 2008). The ICD of both these receptors contains a region known as the Toll/Interleukin-1 receptor (TIR) domain which is conserved among the members of the IL-1R family and can be subdivided into three regions of high similarity (Subramaniam et al., 2004). The mutation in the first two regions prevents signalling from IL-1R1; the mutation in the third region is known to alter the subcellular localization of the protein, preventing its translocation to the cell surface (Slack et al., 2000) **Figure 8.** IL-1R1 and IL-1RAcP have splice variants which function differently and potentiate as well as negatively regulate the IL-1 mediated signalling. IL-1R1 has three reported isoforms, two of which ST2L and sST2 are membrane bound and can transduce IL-1 mediated signals (Li et al., 2000). Additionally, there is a soluble IL-1R1 isoform-vST2, which sequesters IL-1 ligands and attenuates the IL-1 mediated signalling pathway (Tago et al., 2001). There are two IL-1RAcP variants, a membrane bound and a soluble form, that acts to sequester ligand bound IL-1R1 from the cell

surface, which then can no longer interact with IL-1RAcP with full functional capability (Greenfeder et al., 1995; Jensen et al., 2000).

The C-terminal domain of both IL-1R1 and IL-1R1 AcP is required for effective signalling (Huang et al., 1997; Leung et al., 1994). Interestingly, the truncated IL-1R1 lacking the C-terminal domain still binds to IL-1 ligands and IL-1RAcP with the same affinity as the full length IL-1R1. Though this receptor complex undergoes internalization and nuclear localization, it is incapable of undergoing IL-1 mediated signalling (Heguy et al., 1991). This has highlighted the importance of the IL-1R1 C-terminus domain in IL-1 induced signalling pathways. On the other hand, the lack of the N-terminal domain reduces the binding of IL-1 to the receptor by 50% (Heguy et al., 1992). Both IL-1R1 and IL-1RAcP are glycosylated in their N-terminal domain, which is essential for their interaction and the interaction with the ligand (Mancilla et al., 1992). Thus, these data indicate the importance of the N-terminal domain in ligand binding and signalling.

Further investigation into the function of the C-terminal domain of IL-1R1 has revealed that it does not contain any intrinsic protein kinase activity or homology to catalytic subunit of protein kinases (Madge et al., 2000). However, following ligand binding IL-1R1 was shown to undergo phosphorylation and PKC/PKA acceptor sites fitting the consensus KKSRR or R-X-S-T were identified in the C-terminus domain of the receptor; these sites were later shown to be disposable for IL-1 mediated signal transduction (Heguy et al., 1992; Kunos et al., 1993). Furthermore, residues 28-42 in the CTF of the receptor were shown to be necessary for the induction of IL-8 and IL-2 gene expression (Kunos et al., 1993). Extensive mutagenesis studies have been performed and highlight the importance of specific IL-1R1 residues in receptor trafficking and ligand induced signalling **Table 1.6**.

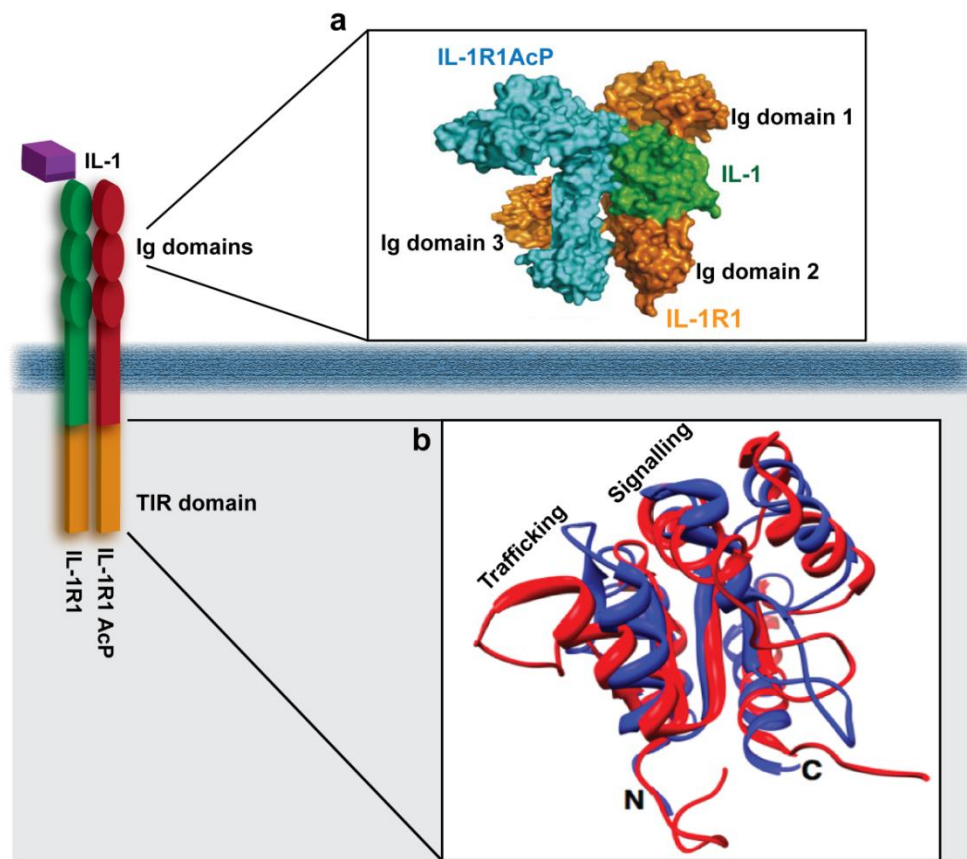


Figure 8 Three-dimensional representation of the Ig and the TIR domains of IL-1R1

(a) Ribbon diagram of Ig domains of IL-1R1 showing the regions interacting with the IL-1 ligand and the IL-1R1AcP. **(b)** Ribbon diagram of IL-1R1 TIR domain, with two regions important for signalling and the third responsible for proper trafficking of the receptor. (Chan et al., 2009; D. Wang et al., 2010)

Table 1.6 Residues mutated and studied in IL-1R1

Residue(s) mutated	Effect	Reference
D369	Defective in NF- κ B activation, stress kinase activation, IL-2 induction under IL-promoter	Slack et al., 2000
Y374	None	Slack et al., 2000
R411	Defective in NF- κ B activation, stress kinase activation, IL-2 induction under IL-promoter	Slack et al., 2000
K428	None	Slack et al., 2000
K429	None	Heuguy et al., 1992
S430	None	Heuguy et al., 1992
R431	None	Slack et al., 2000
R431	No IL-2 induction	Heuguy et al., 1992
K467	None	Slack et al., 2000
E474	None	Slack et al., 2000
K492	None	Slack et al., 2000
K510	None	Heuguy et al., 1992
R512	None	Heuguy et al., 1992
R512A	Trafficking defective	Slack et al., 2000
R512S	Trafficking defective	Slack et al., 2000
F513	Important for IL-2 induction	Heuguy et al., 1992
W514	Trafficking defective, Important for IL-2 induction	Slack et al., 2000; Heuguy et al., 1992
K515	Trafficking defective, Important for IL-2 induction	Slack et al., 2000; Heuguy et al., 1992
R518	Important for IL-2 induction	Heuguy et al., 1992
Y519	Important for IL-2 induction	Heuguy et al., 1992
P522	Important for IL-2 induction	Heuguy et al., 1992
Q524	None	Heuguy et al., 1992
368AAA	Defective in NF- κ B activation, stress kinase activation, IL-2 induction under IL-promoter	Slack et al., 2000
411AAA	Defective in NF- κ B activation, stress kinase activation, IL-2 induction under IL-promoter	Slack et al., 2000
510-512	None (residues not conserved in Toll)	Croston et al., 1995
513-515	Inactive-could not precipitate IRAK or activate NF- κ B (residues conserved in Toll)	Croston et al., 1995
518-520	Inactive-could not precipitate IRAK or activate NF- κ B (residues conserved in Toll)	Croston et al., 1995

1.7.3 IL-1R1 mediated signalling

IL-1 α and IL-1 β induced signalling through IL-1R1 is responsible for the activation of the nuclear factor κ -beta (NF- κ B), and mitogen activated protein kinase (MAPK) signalling pathways (O'Neill et al., 1990; O'Neill, 2008). The MAPK signalling pathways involve three distinct pathways; p42 and p44 [p42/p44; also known as the extracellular signal-regulated kinase (ERK) 1 and ERK 2 (ERK1/2)], Janus N-terminal kinase (JNK) and p38. While ERK1/2 MAPK also appears to regulate transcription, p38 and JNK promote the stabilization of induced mRNA (Chang et al., 2001). These signalling pathways have been extensively studied due to the role played by IL-1 cytokines in inflammatory diseases, including neurodegenerative diseases, atherosclerosis, pulmonary diseases, ischemia induced inflammation, type-2 diabetes, joint and bone diseases, and multiple sclerosis (Lukens and Gross, et al., 2012). Furthermore, IL-1 signalling induces the production of secondary inflammatory cytokines and chemokines such as IL-6, TNF α , KC, and G-CSF (Di Paolo et al., 2009; Orjalo et al., 2009). IL-1 cytokines also lead to inflammatory diseases by promoting the induction of pathogenic cytokines (IFN- γ , IL-17, and GM-CSF) by T cells and innate effector cell (Lukens and Barr, et al., 2012; Sutton et al., 2009).

Despite of the critical role played by IL-1 mediated signalling pathways; there are fewer than 100 IL-1R1 proteins on most cell surfaces at any one time and occupancy of as little as 5% is sufficient for IL-1 signal transduction (Arend et al., 1990). Several contradictory outcomes have been reported regarding IL-1R1 signalling pathways. This reflects cell type- and species specific responses to IL-1. NF- κ B activation can be mediated through two separate pathways- transforming growth factor- β -activated protein kinase 1 (TAK1)-independent or TAK1-dependent pathway (Yao et al., 2007). Each of these pathways is distinct from the other based on the subcellular site for the signal transduced. Unlike the TAK-1 dependent pathway, the TAK-1 independent

pathway is initiated from the cell surface and does not require internalization of IL-1R1 ligand complex (Takaesu et al., 2001).

1.7.3.1 TAK1-independent pathways-mediated at the cell surface

It is well established that IL-1 stimulation leads to the recruitment of IL-1RAcP forming the IL-1/IL-1R1/IL-1RAcP complex (Greenfeder et al., 1995; Huang et al., 1997). The TIR domains of these two receptors act as a scaffolding platform for further recruitment of MyD88. The MyD88 recruitment leads to the recruitment of interleukin-1 receptor-associated kinase (IRAK)1 and IRAK4, via their death domains (DD) (Zhaodan Cao et al., 1994; Loiarro et al., 2009; Wesche et al., 1997). IRAK4 then activates IRAK1, which undergoes autophosphorylation which in turn interact with TRAF6, an E3 ubiquitin ligase (Kollewe et al., 2004; Li et al., 1999).

At this stage two different signalling pathways are initiated leading to NF- κ B activation. The first pathway involves the recruitment of mitogen-activated protein kinase kinase 3 (MEKK-3) (Yao et al., 2007). On the other hand, the second pathway requires the interaction of ubiquitinated TNFR-associated factor-6 (TRAF6) with p62 leading to activation of atypical PKC (aPKC) (Geetha et al., 2002; Sanz et al., 2000). Both these events are followed by phosphorylation of IKK β leading to the activation of IKK α . Upon activation IKK α phosphorylates I κ B α such that it is not recognized by SCF-bTrCP, thus preventing its proteasome-dependent degradation while it is associated with NF- κ B, following which the phosphorylated I κ B α dissociates from NF- κ B, thus allowing NF- κ B to translocate to the nucleus (Solt et al., 2010) **Figure 9**.

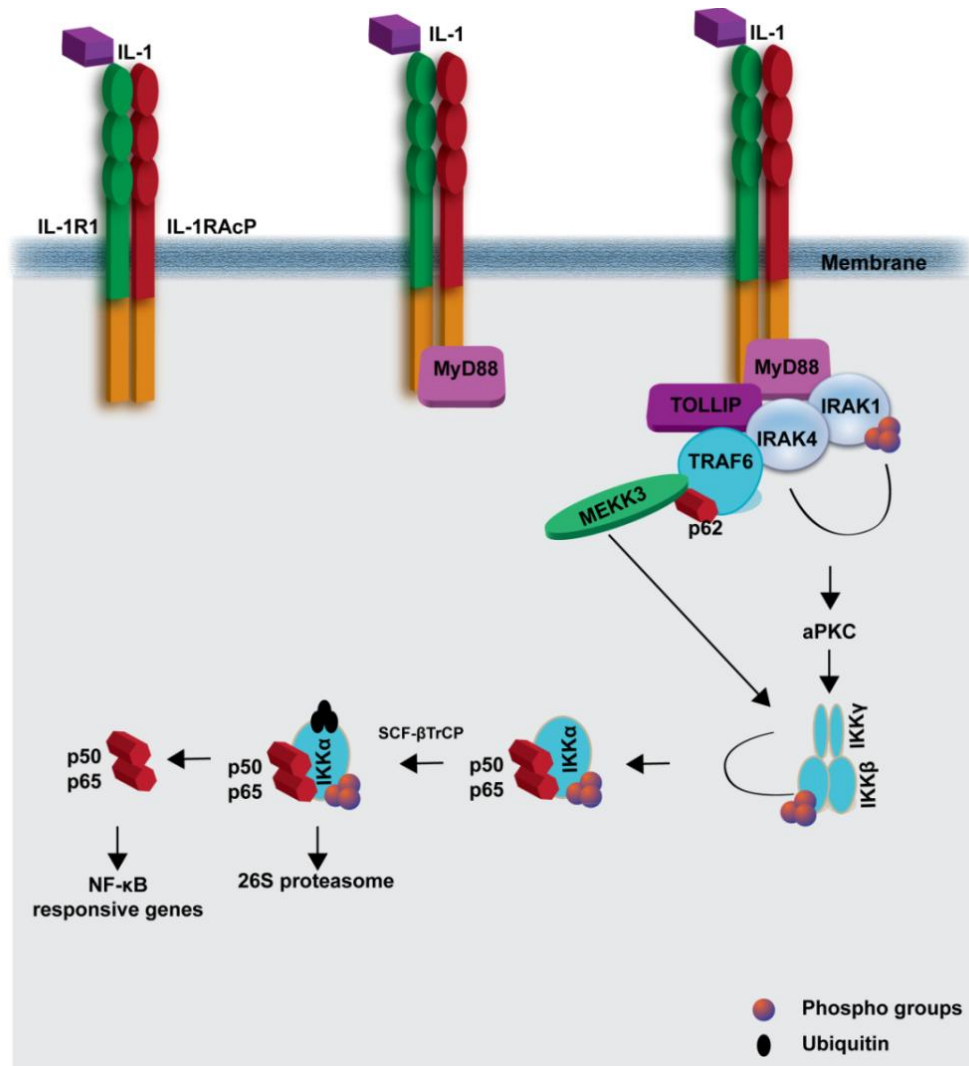


Figure 9 TAK-1-independent pathways

IL-1 binds to IL-1RI forming a complex with IL-1RAcP, leading to recruitment of MyD88, Tollip, IRAK-1, IRAK-4 and TRAF6 to form complex I. TRAF6 then interacts with p62, leading to activation of atypical PKCs and phosphorylation of IKK β leading to activation of IKK α which subsequently phosphorylates I κ B α in a way that does not trigger its recognition by SCF-bTrCP and proteasome- dependent degradation. Phosphorylated I κ B α dissociates from NF- κ B, thus allowing NF- κ B to translocate to the nucleus. Alternatively, TRAF6 can also interact with MEKK3, leading to activation of IKK α , and subsequently NF- κ B (Adapted from Verstrepen et al., 2008).

1.7.3.2 TAK1-dependent pathway

The formation of the IL-1/IL-1R1/IL-1RAcP complex leads to the recruitment of MyD88, Tollip, IRAK-1, IRAK-4 and TRAF6 to form complex I (Jiang et al., 2002). Formation of complex I leads to the activation and autophosphorylation of IRAK-1, making it available as an adaptor protein in IL-1 signalling pathway (Kollewe et al., 2004). MyD88 binding induces endocytosis of IL-1R1 following ligand binding (Li et al., 2006), which is followed by endosomal dissociation of MyD88 and Tollip, but not TRAF6. After dissociation from the complex I, IRAK-1/TRAF6/IRAK-4 complex interacts with the pre-existing membrane bound Transforming growth factor β -activated protein kinase 1 (TAK1)-binding protein (TAB) (TAK1-TAB1-TAB2/3) complex, forming complex II. IRAK-1 undergoes K-63 linked poly-ubiquitination by Pellino, which is itself phosphorylated by IRAK-1 (Ordureau et al., 2008). Following which both IRAK-1 and Pellino undergo K-48 linked polyubiquitination and are degraded (Ordureau et al., 2008; Yamin, 1997). The TRAF6-TAK1-TAB1-TAB2/TAB3 complex then leaves the membrane and is referred to as complex III (Jiang et al., 2002). While in the cytoplasm TRAF6 undergoes auto-ubiquitination and activates TAK1/TAB2 (Walsh et al., 2008). These events are followed by phosphorylation of IKK β which then lead to the activation of IKK α . IKK α upon activation phosphorylates I κ B α such that it is not recognized by SCF-bTrCP, thus preventing its proteasome-dependent degradation (Cui et al., 2012). Following this, the phosphorylated I κ B α dissociates from NF- κ B, thus allowing NF- κ B to translocate to the nucleus and binds to promoters of responsive genes (Solt et al., 2010) **Figure 10.**

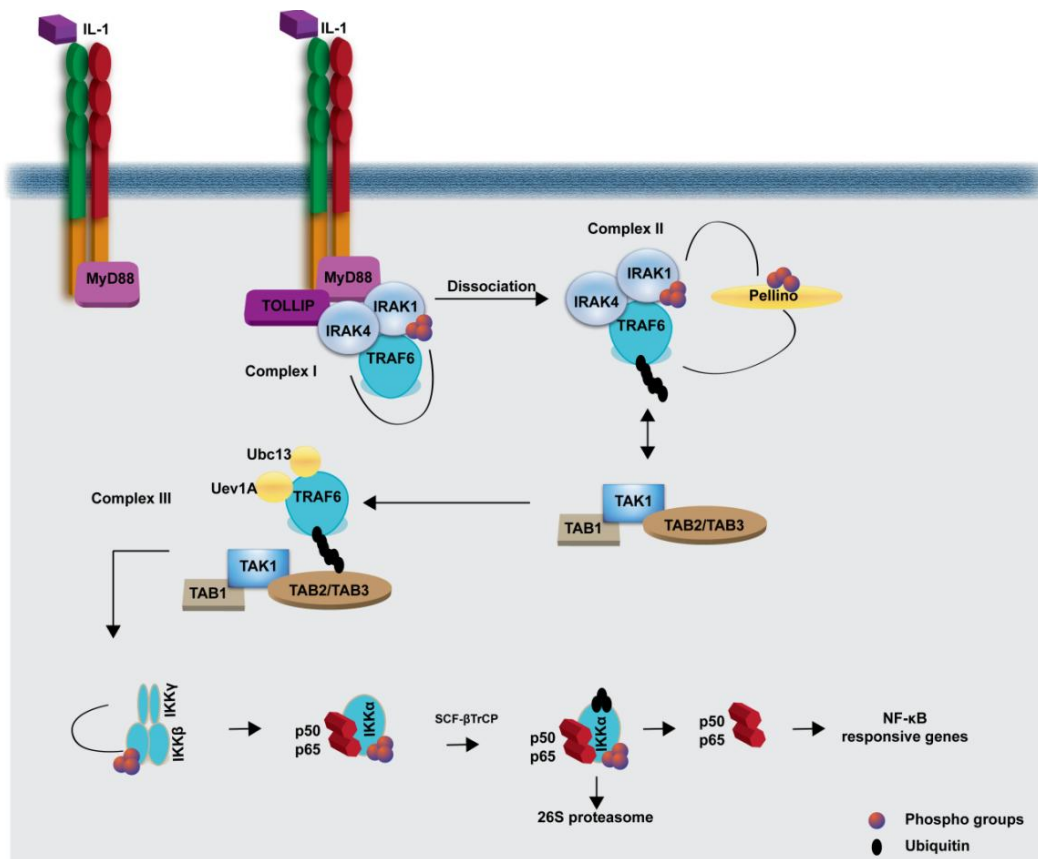


Figure 10 TAK-1-dependent pathways

The formation of IL-1R complex leads to the recruitment of MyD88, Tollip, IRAK-1, IRAK-4 and TRAF6 to form complex I. This then triggers IRAK-4 autophosphorylation and the phosphorylation of IRAK-1. Subsequently, IRAK-1 leaves the receptor complex together with TRAF6 and associates with the pre-formed TAK1-TAB1-TAB2/TAB3 complex (complex II). A series of events lead to the release of TRAF6-TAK1-TAB1-TAB2/TAB3 from the membrane (complex III). In the cytoplasm, TRAF6 undergoes auto-ubiquitination which triggers the binding of TAB2 and activation of TAK1, which subsequently phosphorylates IKK β . IKK β phosphorylates I κ B α followed by proteasome-dependent degradation. In this way, NF- κ B (shown as p65/p50 dimers) is set free and translocates to the nucleus to bind the promoters of responsive genes (Adapted from Verstrepen et al., 2008).

1.7.4 Regulation of IL-1R1 signalling

IL-1R1 signalling is regulated at several steps, beginning from the controlled expression of IL-1R1 on the cell surface, to the existence of the IL-1Ra which has a high affinity to IL-1R1 but fails to recruit IL-1RAcP and induce a signal. Additionally, another receptor, IL-1R2 is referred to as the decoy receptor since ligand binding to IL-1R2 does not result in cytokine production (Garlanda et al., 2013). While IL-1R2 shares sequence homology to IL-1R1, it lacks the C-terminal domain, making it incapable of IL-1 induced signal transduction (Mcmahan et al., 1991). Thus, IL-1R2 acts by sequestering IL-1 ligands and dampening the IL-1R1 signalling cascade. Certain viruses such as Vaccinia virus or cowpox have been shown to encode genes for proteins with 30% homology to IL-1R2. These viral mimics of IL-1R2 diminish the host's ability to mount an inflammatory response, allowing the viruses to replicate at a higher rate (Alcamí et al., 1992; Spriggs et al., 1992).

The co-ordinated regulation of IL-1R1, IL-1R2 and IL-1Ra represents a principal mechanism involved in controlling IL-1 local effects (Bellehumeur et al., 2009). Furthermore, sIL-1R1 has higher affinity towards the IL-1Ra than the sIL-1R2 (Svenson et al., 1993). Furthermore, the inhibitory activity of IL-1Ra is enhanced by sIL-1R1 and diminished by sIL-1R2 (Burger et al., 1995). Thus it is feasible that the extracellular domain of IL-1R1 functions to sequester the IL-1Ra which allows the IL-1 ligands to bind to IL-1R1 efficiently and transduce a signal across the membrane. Also reports indicating the presence of a soluble splice variant of the IL-1R2 encoding its extracellular domain functions to sequester ligands from the extracellular matrix, causing a negative effect on IL-1 signalling (Cui et al., 2003; Hart, 1996).

Apart from triggering the formation of IL-1R1/IL-1RAcP complex, IL-1 β , also induces endocytosis of IL-1R1. Upon internalization activated receptor complexes are often sorted to late endosomes and targeted for degradation (Goh et al., 2013). However,

the IL-1R1 receptor complex has been reported to stay in cells without getting processed or degraded for up to 6 hours, after which it translocates to the nucleus (Curtis et al., 1990). Interestingly, nuclear localization signal sequences within the intracellular fragment of IL-1R1 are dispensable for IL-1 β induced signal transduction and translocation of the IL-1R1-IL-1 β complex to the nucleus. Also Heguy and co-workers constructed a series of deletion mutants of the cytoplasmic domain of the IL-1R1 and showed that these mutants internalized and trafficked to the nucleus, but failed to induce IL-2 (Heguy et al., 1992). Furthermore, they showed that the amino acids 527-477 were required to transduce signals mediating activation of IL-2 or SV40 promoter activation (Heguy et al., 1991). Furthermore, Brissoni and co-workers showed that IL-1R1 interacted with Tom1, an ubiquitin-, clathrin, and Tollip-binding protein; knockdown of Tom1 resulted in IL-1R1 accumulation in the late endosome. This group also suggested that Tollip is an endosomal adaptor protein linking IL-1R1, via Tom1, to the endosomal degradation pathway (Brissoni et al., 2006). Furthermore, Tollip deficient mice produce less TNF α and IL-6 after IL-1 β administration, despite the absence of any defects in I κ B, JNK, p38 and ERK signalling pathways (Didierlaure et al., 2006). Thus, these data clearly indicate an important role of trafficking of IL-1R1 trafficking and the signalling pathways activated.

Our group previously showed that IL-1R1 undergoes ectodomain shedding on stimulation with IL-1 β or the PKC activator- PMA. IL-1R1 was shown to undergo ectodomain shedding by an unidentified metalloprotease, releasing the sIL-1R1 fragment into the extracellular matrix. The IL-1R1 CTF generated was subsequently shown to be cleaved by γ -secretase enzyme complex. Additionally, the inhibition of γ -secretase-mediated cleavage of IL-1R1 was found to negatively regulate IL-1 β responsiveness (Elzinga et al., 2009), however, the mechanism underlying this regulation remains unclear. Taking a closer look at the mutagenesis studies carried out by several groups over the past years, specifically by Heguy and co-workers in the ICD

of IL-1R1, it is possible that the ectodomain shedding and subsequent γ -secretase-mediated cleavage of IL-1R1 functions to regulate IL-1-mediated-signalling at a completely different level.

1.8 Tumor necrosis factor superfamily

In 1891, Dr. William Coley performed an experiment with an intention to treat cancer in a terminally ill patient. He injected the man with a solution of infectious bacteria known to cause a skin disease called erysipelas. He expected that the patients while recovering from the skin disease would also recover from cancer. It was an experiment and he was successful in treating cancer in terminally ill patient (Coley., 1893). The toxin injected by William Coley was called Coley's toxin for a long time. In 1984 in an attempt to isolate Coley's toxin, Aggarwal and co-workers discovered two cytotoxic factors, one from macrophages and the other from lymphocytes with 50% sequence homology. These toxins came to be known as Tumor Necrosis Factor- α (TNF α) and lymphotoxin- α (LT α), which was later renamed TNF- β (Aggarwals et al., 1985). They were the first discovered members of the TNF superfamily. Over the past three decades, the TNF superfamily has expanded and now consists of over 19 different cytokines, 29 interacting receptors (three additional TNF superfamily receptors have been identified and found only in mice) and several adaptor proteins. The main TNF superfamily death ligands and their receptors are shown in **Figure 11**. The TNF receptors are oligomeric, type I or type III transmembrane proteins which can be divided into two groups, based on the presence or absence of the intracellular death domain (DD). The DD consists of 45 amino acids and is essential for the interaction and recruitment of caspase-interacting proteins following ligand binding to initiate the extrinsic pathway of caspase activation, thereby transducing cell death signals (Feinstein et al., 1995; Tartaglia et al., 1993). TNF receptors lacking the DD bind TNF receptor-associated factors (TRAFs) and activate intracellular signalling pathways that can lead to cell proliferation or differentiation (Arch et al., 1998; Gilfillan, 1996; Rothe

et al., 1995). The extracellular domain of these receptors contains multiple cysteine-rich domains.

1.8.1 Tumor necrosis factor receptor-1 (TNFR1)

In 1990 TNFR1 was first isolated and characterised (Loetscher et al., 1990). TNFR1, also known as CD120a, p55/p60 and death receptor 1 (DR1), is a type I transmembrane protein. The extracellular domain of TNFR1 includes four-40 amino acids long cysteine-rich domains (CRDs) (CRD1 or the pre-ligand binding assembly domain (PLAD), CRD2, CRD3 and CRD4) involved in ligand binding (Marsters et al., 1992). The cytoplasmic domain of TNFR1 has the characteristic DD which acts as a scaffolding site for recruitment of the adaptor proteins and the subsequent formation of the death inducing signalling complex (DISC) (Tartaglia et al., 1993). The importance of TNFR1-mediated signalling is highlighted by the TNFR1 knock out animals. These animals show a defective immune phenotype with abnormal microglial cell morphology, decreased inflammatory response, decreased susceptibility to endotoxin shock, increased circulating TNF levels, decreased IL-6, increased susceptibility to bacterial infection and lung inflammation (Iosif et al., 2006; Kanehiro et al., 2002; Peschon et al., 2013). Apart from this, these animals also show decreased tumor incidence (Arnott et al., 2004). Thus, these data indicate a role for TNFR1 in immune signalling and cell death regulation.

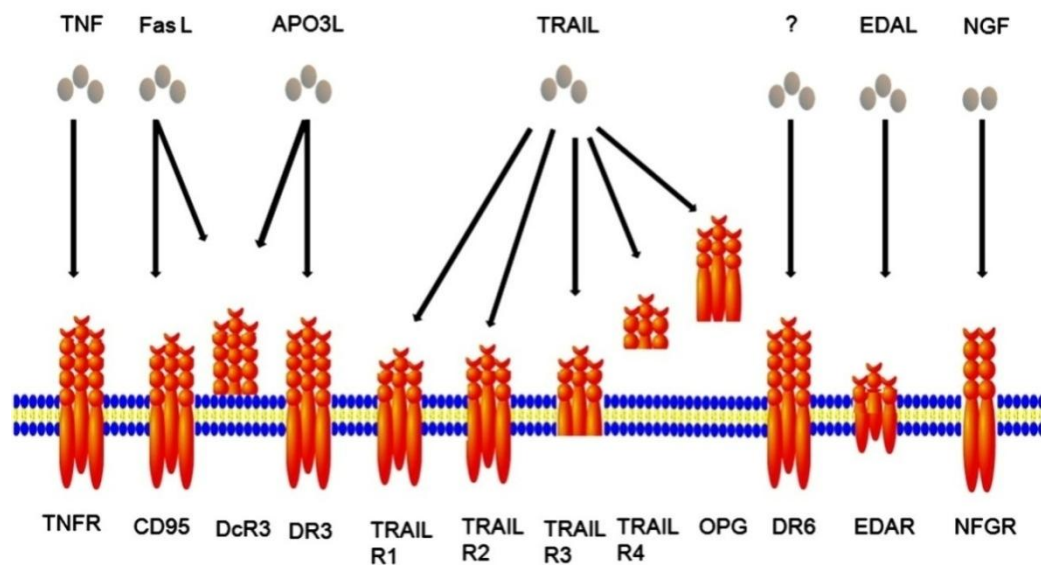


Figure 11 Death ligands and their receptors

The figure shows the known TNF superfamily death ligands and their receptors (Mahmood et al., 2010).

1.8.2 TNFR1 compartmentalization and signalling

TNFR1 binds to the TNF ligand, which leads to the trimerization of the receptor-ligand complex. This is followed by cleavage in its extracellular domain by a family of sheddases, namely ADAM10 or ADAM17, releasing the extracellular domain of the receptor into the extracellular milieu (Edwards et al., 2008). The TNFR1 DD then provides a platform for the recruitment of the adaptor protein TNF receptor 1-associated protein (TRADD) via the homotypic DD interactions. TRADD then recruits TRAF2, RIP1 and other associated proteins-UBC13, cIAP1/2 (Zarnegar et al., 2008). The complex thus formed at the cell surface is referred to as the Complex I which signals for NF- κ B activation. There is a lot of discrepancy regarding the adaptor proteins recruited to the Complex I, and the order in which they are recruited. This has been attributed to the cell type and species specific differences. A general consensus about the adaptor proteins recruited to form the two complexes is highlighted in **Figure 12**.

The TNFR1 complex I then undergoes internalization and gets dissociated. This event leads to the formation of endosomal complex II. It has been reported by several groups that two different types of complex II referred to as complex IIA and complex IIB can be formed depending upon the adaptor proteins recruited (Gyrd-Hansen et al., 2010). Though there has been a lot of discrepancy about the spatial segregation of these complexes, the subcellular fractionation studies and studies reporting that the formation of complex II is enhanced upon knock-out or knockdown of components of complex I, has validated the compartmentalization of TNFR1 mediated signalling (Hsu et al., 1996; Schneider-Brachert et al., 2004).

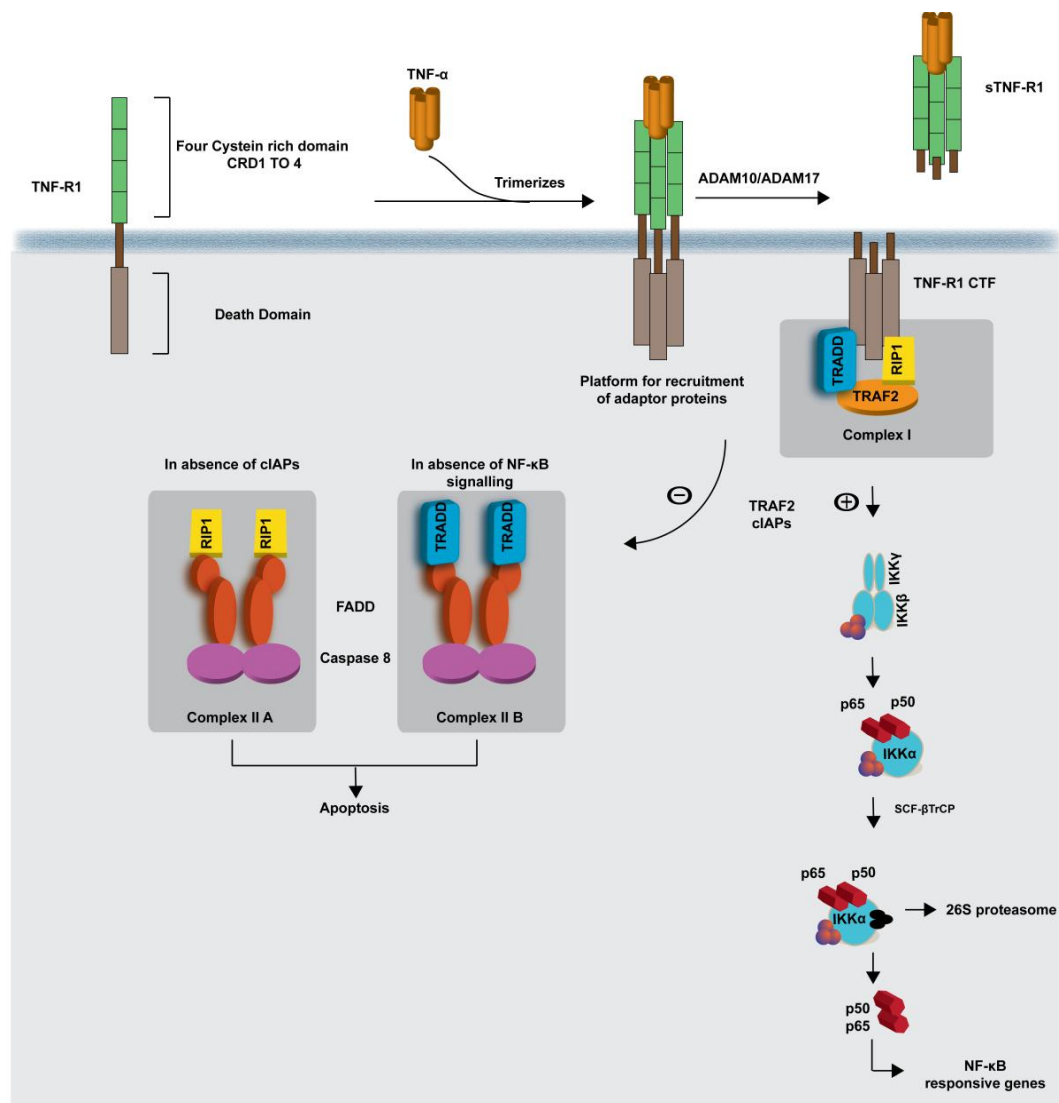


Figure 12 Compartmentalization of TNFR1 signalling pathways

Upon TNF stimulation, TNFR1 trimerizes and recruits TRADD via its DD. TRADD in turn binds to TRAF2 and RIP1. In absence of NF-κB signalling following TNFR1 endocytosis, TRADD dissociates from TNFR1 and associates with FADD and Caspase-8 activating the pro-apoptotic pathway. Alternatively in absence of cIAPs, RIP1 dissociates from TNFR1 following endocytosis. RIP1 then recruits FADD and Caspase 8, leading to the activation of pro-apoptotic pathways (Adapted from Verstreppe et al., 2008; Gyrd-Hansen and Meier, 2010).

Complex IIA is formed in the absence of NF- κ B signalling. In Complex IIA, upon endocytosis TRADD dissociates from TNFR1 and associates with Fas-Associated protein with Death Domain (FADD) which then recruits Caspase 8, thereby initiating the pro-apoptotic signalling. Interestingly, in the absence of cIAPs it is RIP which dissociates from the TNFR1 complex I, recruiting FADD and Caspase 8 forming Complex IIB. This complex initiates the pro-apoptotic signalling pathways. Thus, TNFR1 can initiate both pro-survival and pro-apoptotic signalling pathways.

1.8.3 TNFR1 mutational studies

To characterize TNFR1 and better understand the role of the intracellular and extracellular domains in terms of ligand binding and the signalling pathways mediated, several TNFR1 deletion mutants and point mutants have been made **Table 1.7**.

Table 1.7 Residues mutated and studied for the TNFR1

Residue(s) mutated/deleted	Effect	Reference
Δ 218	Inhibition of cytotoxic signals	Tartaglias and Goeddel, 1992
L351	Critical for induction of necrosis	Boone et al., 2000
Δ 405-413	Important for localization of TNF-R1 to lipid rafts	Cottin et al., 2002
Δ 218-234	Negative regulation of cell surface expression	Fielding et al., 2004
W239	Required for clathrin mediated internalization	Schneider-Brachert et al., 2004

Deletion mutagenesis of the cytoplasmic domain identified an ~80 amino acid domain (Δ 218) responsible for signalling cytotoxicity (Tartaglia et al., 1992). Following which attempts were made to map the region responsible for downstream signalling. Several of the residues within this deleted region were conserved with the Fas antigen. Mutation in any of these residues was shown to inhibit cytotoxic signals (Tartaglia et al., 1993). Further mutagenesis studies showed that the lysine residue numbered 351 was critical for induction of necrosis (Boone et al., 2000). Following this discovery, studies were carried out to further characterize these signalling pathways in terms of

spatial signal segregation. The domain corresponding to amino acids 405-413 were shown to be important for the localization of TNFR1 to lipid rafts (Cottin et al., 2002). In this context, C.A. Fielding and co-workers pointed out that residues 218-324 negatively regulated cell surface expression of TNFR1 (Fielding et al., 2004). This domain was referred to as TNFR1 internalization domain (TRID). This internalization site was then mapped to tryptophan residue 239, which was responsible for clathrin mediated internalization of the receptor (Schneider-Brachert et al., 2004). This group thus established the spatially segregated TNF signalling pathways by compartmentalization of plasma membrane-derived endocytic vesicles harbouring the TNFR1-associated DISC.

1.9 Research Objective

Regulated intramembrane proteolysis is a two-step sequential process where ectodomain shedding has been regarded as the key regulatory event for the further processing of proteins by the γ -secretase protease complex. The importance of ectodomain shedding and γ -secretase-mediated cleavage is highlighted by the increasing list of their substrates. Studies carried out to determine the expression pattern, substrates and the subsequent direct or indirect involvement of these enzymes in various signalling pathways has allowed future progress to target these enzymes for various diseases. The subcellular location for the activity of sheddases varies from Golgi vesicles to the plasma membrane and shed microvesicles. Furthermore, ectodomain shedding is regulated by several factors including signalling pathways and post-translational modifications. Apart from direct regulation of the sheddases, spatial segregation of its substrates is another mode of regulation. Also, the γ -secretase enzyme complex is active on cell surface, endosomes, ER, and *trans*-Golgi. Thus, it is important to study the subcellular location and trafficking of γ -secretase substrates and the fragments generated post cleavage to define the role they might play in the signalling events mediated and their regulation.

We previously reported IL-1R1 to undergo ectodomain shedding followed by γ -secretase-mediated regulated intramembrane proteolysis. Additionally, we also highlighted a role for TRAF6 in regulated intramembrane proteolysis of IL-1R1, where TRAF6-mediated ubiquitination was shown to positively modulate regulated intramembrane proteolysis of IL-1R1 (Twomey et al., 2009). Recent studies reflect the importance of trafficking and degradation of γ -secretase cleavage products in the regulation of cell signalling, and also emphasize the role played by ubiquitination in mediating subcellular trafficking of proteins. However nothing is yet known about the subcellular location for ectodomain shedding and regulated intramembrane proteolysis of IL-1R1 or how these processes are regulated. Thus, in this research study

we focused on the subcellular occurrence of IL-1R1 cleavage events and studied the role played by TRAF6 in mediating IL-1R1 proteolysis and determined the fate of the IL-1R1 ICD. The second main aim of this work is to determine the conservation of regulated intramembrane proteolysis as a physiological occurrence amongst other immune receptors. In this regard, similar to Interleukin 1, receptor, type 1, (IL-1R1), we examined the Toll like receptor 4 (TLR4) as a potential γ -secretase substrate. Furthermore we determined the role of PS proteins in apoptosis by screening cell death receptors as potential γ -secretase substrates. The role of PS proteins in apoptosis was highlighted when conditional deletion of *Psen1/Psen2* in adult mouse brains led to progressive loss of synapses, dendrites, and neurons, suggesting that loss of *Psen* function, caused neuronal degeneration which was credited to increased apoptosis. This phenotype has always been attributed to defective Notch signalling following inhibition of γ -secretase activity (Curry et al., 2005; Rosati et al., 2013). However, the involvement of specific cell death receptors as potential γ -secretase substrates has not been studied, highlighting the need to find potential γ -secretase substrates in the cell death receptor family. In this context TNFR1 was investigated as a potential γ -secretase substrate. We also studied the subcellular occurrence of the cleavage events for TNFR1, and the role played by this cleavage on both signalling and apoptosis.

Chapter 2

Material and Methods

2.1 Materials

2.1.1 General Chemicals and Reagents

All salts and reagents were purchased from Sigma-Aldrich (Dublin, Ireland) unless otherwise stated. Protein G-sepharose beads and DNA ladder were procured from Invitrogen (Paisley, Scotland). Prestained molecular weight protein marker was purchased from BioRad Laboratories (GmbH, Munich, Germany). Bicinchoninic acid (BCA) protein concentration reagents were purchased from Pierce Biotechnology (Rockford, Illinois) through Medical Supply Company (MSC) Ltd. (Dublin, Ireland). Nitrocellulose membrane and western blotting filter paper were purchased from Schleicher and Schuell (GmbH, Dassel, Germany). The chemiluminescent detection kit ECL was purchased from GE Healthcare (Buckinghamshire, UK). Complete protease inhibitor tablets were purchased from Roche (Boehringer-Mannheim, Indianapolis, USA). Recombinant murine IL- β and TNF α was purchased from Peprotech (Dublin, Ireland). Phorbol 12-myristate 13-acetate (PMA), recombinant human TNF α , recombinant human IL-1 β and LPS was procured from Calbiochem (Nottingham, England) **Appendix Table 1.**

2.1.2 Molecular Biology Reagents

Primers were ordered and synthesised by Sigma-Aldrich (Dublin, Ireland) or Integrated DNA Technologies (Leuven, Belgium). DNA polymerase and KOD hot start DNA polymerase were purchased from Calbiochem (Nottingham, England). High-fidelity Pfu polymerase was bought from Stratagene (Edinburgh, Scotland). T4 DNA ligase and all restriction enzymes were purchased from New England Biolabs through ISIS (Co. Wicklow, Ireland). PCR and DNA Gel purification kits were obtained from Qiagen Ltd. (West Sussex, UK). DNA midiprep kit and Opti-MEM or Turbofect transfection medium were provided by Promega through Millipore (Southampton, UK). 4',6-diamidino-2-

phenylindole (DAPI) and Prolong gold antifade mounting media were purchased from Invitrogen, Ireland. Glass coverslips were purchased from Reagecon, UK. **Appendix Table 1**

2.1.3 Plasmid Sources

FLAG-tagged TNFR1 was a gift from Dr. Martin S. Kluger (Yale University School of Medicine, Connecticut). IL-1R1 and FLAG-tagged TRAFs constructs were kindly provided by Dr. Vishva Dixit (Genetech, South San Francisco, CA). HA-tagged Ubiquitin constructs were obtained from Dr. Ruaidhri Carmody (University of Glasgow). HA-tagged Ubiquitin K48 only and K63 only were provided by Dr. Mathijis Baens (University of Leuven, Leuven, Belgium). Dr. Aymelt Itzen (Max-Planck-Institute for Molecular Physiology, Dortmund, Germany) kindly provided the GFP-tagged Rab5 constructs and GFP-tagged Rab7 constructs were a gift from Dr. Cecilia Bucci (Department of Science and Technology, University of Salento, Italy). The wild-type Dynamin and Dynamin-K44A mutant was procured from Dr. Pietro De Camilli (Addgene 22163 and Addgene 22197). All the other constructs were generated in-house.

Appendix Table 2

2.1.4 Vector Maps

Vector maps for the constructs are listed in the **Appendix Table 3**. These include pcDNA3, pBabepuro, pRK5, pEGFP-C1, pEGFP-N1, pEYFP-C1, and pEYFP-N1.

2.1.5 Primers

Primers are listed in the tables below for each construct generated and used in the study.

Mutation	Primer	Sequence
TNF-R1 W239A	Forward Reverse	3'-CGC TAC CAA CGG GCG AAG TCC AAG CTC -5' 3'- GAG CTT GGA CTT CGC CCG TTG GTA GCG -5'
IL-1R1 W262A	Forward Reverse	3'-GGA CTC AGG CAC CAC AGC GGC GTT GCC CCT GGT C-5' 3'- GGG GCA ACG CCG CTG TGG TGC CTG AGT CCT CAG TGC -5'
IL-1R1 Y278A	Forward Reverse	3'-GGC ACC ACA GTG CTG TTG GCC GCG GTC ATT TTC TTT GGT C -5' 3'- GGC AAA GAC CAA AGA AAA TGA CCG CGG CCA ACA GCA CTG -5'
IL-1R1 K360R	Forward Reverse	3'-GCT GTT GCC CCT GGT CGC TGC CTT TGG TCT TTG CC -5' 3'- CCA AAG GCA GCG ACC AGG GGC AAC AGC ACT GTG GTG CC 5'
IL-1R1 K378R	Forward Reverse	3'-GCC CCT GGT CAT TTT CTT TGC TGC TTG CCT TTT ATC CC -5' 3'- GAG GGA TAA AAG GCA AGC AGC AAA GAA AAT GAC CAG GG 5'
IL-1R1 K383R	Forward Reverse	3'-GGT CTT TGC GCT GCA TCC CTC CTC TTC ATT GG -5' 3'- GAG GAG GGA TGC AGC GCA AAG ACC AAA GAA AAT G -5'
IL-1R1 K445R	Forward Reverse	3'-GCC TTT TAT CCG CCG CCT TCA TTG GTT TAA TGT ATC GC -5' 3'- CCA ATG AAG GCG GCG GAT AAA AGG CAA AGA CC -5'
IL-1R1K446R	Forward Reverse	3'-GCC TTT TAT CCC TCC TCT TCG CTG CGT TAA TGT ATC GC -5' 3'- GCG ATA CAT TAA CGC AGC ACC AAT GAA GAG GAG GG -5'
IL-1R1 K527R	Forward Reverse	3'-CCT CTT CAT TGG TTT AGC GGC TCG CTA CCA ACG G -5' 3'- GCG AGC CGC TAA ACC AAT GAA GAG GAG GGA TAA AAG GC -5'
IL-1R1 K532R	Forward Reverse	3'-GCC TTT TAT CCG CCG CCT TCA TTG GTT TAA TGT ATC GC -5' 3'- CCA ATG AAG GCG GCG GAT AAA AGG CAA AGA CC -5'
IL-1R1 K548R	Forward Reverse	3'-GCC TTT TAT CCC TCC TCT TCG CTG CGT TAA TGT ATC GC -5' 3'- GCG ATA CAT TAA CGC AGC ACC AAT GAA GAG GAG GG -5'

2.1.6 Antibodies

Primary Antibodies

The following antibodies were used in the study: anti-TNFR1 (S-20), anti-TNFR1 (N-20), anti-TNFR1 (H-80), anti-IL-1R1 (C-20), anti-IL-1R1 (N-20), anti-TLR4 (C18), anti-YFP, anti-TRAF6, anti-TRADD, anti-I κ B α and anti-JNK were purchased from Santa Cruz, CA, USA. Anti-human PS1-NTF and anti-human PS1-CTF were purchased from Chemicon (Hampshire, UK); PS1 antibody (3.6.1) was previously described (Kirschenbaum et al., 2001). Anti-TNFR1 (C25C1), anti-RIP1, anti-PS2, anti-phospho I κ B α and anti-phospho JNK were obtained from Cell Signalling Technology (Danvers, Massachusetts, USA). Anti-HA antibody was purchased from Covance (Berkely, California, USA). Antibodies purchased from Abcam (Cambridge, UK) included anti-Histone 2b, anti-Na⁺/K⁺ ATPase, anti-Rab5, anti-GFP and anti-EEA1 antibody was from BD Biosciences (Oxford, UK). Anti- β -actin, anti- β -tubulin, anti-FLAG and anti-FLAG FITC antibody were from Sigma (Wicklow, Ireland).

Isotype Antibodies

Anti-mouse IgG, anti-rabbit IgG and anti-goat IgG were purchased from Santa Cruz (CA, USA).

Secondary Antibodies

Peroxidase-conjugated secondary anti-rabbit, anti-mouse and anti-goat antibodies were purchased from DAKO (Denmark). Infrared secondary antibodies- IRDye 680 Goat Anti-Rabbit IgG and IRDye 800CW Goat Anti-Mouse IgG were procured from LI-COR (Dublin, Ireland). Alexa Fluor[®] anti-rabbit 488, Alexa Fluor[®] anti-rabbit 546, Alexa Fluor[®] anti-mouse 488, Alexa Fluor[®] anti-mouse 546, Alexa Fluor[®] anti-goat 488 and Alexa Fluor[®] anti-goat 546 were procured from Invitrogen (Dublin, Ireland). The

catalogue number and the dilution used for the antibodies are listed in the **Appendix Table-4**.

2.1.7 Pharmacological modulators of protease activities

Sodium orthovanadate, dithiothreitol (DTT), leupeptin, ethylene glycol bis (2-aminoethyl ether) tetra acetic Acid (EGTA) and ethylene diamine triacetic acid (EDTA) were purchased from Sigma-Aldrich (Dublin, Ireland). Phorbol 12-myristate 13-acetate (PMA), in solution™ γ -Secretase Inhibitor (XIX), in solution™ TAPI, DAPT, epoxomicin and concanamycin were purchased from Calbiochem (Nottingham, England). **Appendix Table 1**

2.1.8 Cell lines

Human embryonic kidney 293T (HEK293T) cells originally purchased from ATCC (Middlesex, UK) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 1% (v/v) L-glutamine, 50 units/ml penicillin and 50 g/ml streptomycin. Immortalized fibroblasts derived from PS1-deficient, PS2-deficient or both PS1-and PS2-deficient or wild-type mouse embryos were generously provided by Bart De Strooper (KU Leuven, Belgium) and previously described (B De Strooper et al., 1998). All murine embryonic fibroblast (MEF) cell lines were grown in DMEM, 10% (v/v) FBS, 1% (v/v) glutamine, 1% (v/v) non-essential amino acids, 1% (v/v) sodium pyruvate, penicillin (50units/ml) and streptomycin 5g/ml. All cell lines were grown in a humidified incubator at 37°C and 5% CO₂.

2.1.9 Bacterial Strains and Media used

E. coli DH5 α was purchased from Invitrogen (Dublin, Ireland). Lab stocks of the *E. coli* DH5 α strain were grown in pre-made Luria-Bertani (LB) broth.

2.2 Methods

2.2.1 Computer software used in Bioinformatics analysis

DNA sequence analysis and alignments were performed using Vector NTI. Bioinformatics analysis was carried out using the online tools PredictNLS and NetNES to detect NLS and NES respectively. NetNES is available online at <http://www.cbs.dtu.dk/services/NetNES/> and PredictNLS at <http://cubic.bioc.columbia.edu/cgi/var/nair/resonline.pl>.

2.2.2 Competent cell preparation and transformation

DH5- α strain of *E.coli* grown to log or exponential phase was streaked for single colonies on LB agar plate and grown overnight at 37°C. A single colony was picked and inoculated in 5ml of LB broth medium overnight at 37°C, 250rpm/min. Then 3ml of overnight grown culture was inoculated into 100-200ml of LB broth solution and grown for 2-3h. Once cell density reached a value of 0.6 to 1.0 at OD595, cells were harvested by centrifugation at 3000x g for 15min at 4°C. The supernatant was discarded and the pellet resuspended in pre-cooled 0.1 M MgSO₄ at one third of the bacterial culture volume. Cells were then harvested as above and the pellet resuspended in 0.1M CaCl₂ containing 15% (v/v) of glycerol with 1/25 of bacterial culture volume. Aliquots were put in pre-cooled tubes, frozen on dry ice and then stored at -80°C.

2.2.3 Transformation of DH5 α competent cells

Competent cells were thawed on ice for 15-20min. Approximately 1ng of a plasmid DNA or 2-18 μ l of a ligation mixture was mixed with 100 μ l of competent cells and incubated on ice for 30min. Cells were heat-shocked at 42°C for 45sec and immediately cooled on ice. Then 900 μ l of LB broth was added and the culture was incubated at 37°C with shaking for 1h. Subsequently 100-200 μ l of the cell suspension

was spread on a pre-warmed LB agar plate containing an appropriate antibiotic (50µg/ml ampicillin or 50 µg/ml kanamycin) and incubated at 37°C overnight. A single colony was picked and inoculated in 5ml LB broth medium, shaking at 37°C for 6-8h. Then 1ml of the inoculated media was incubated shaking at 37°C overnight in 200ml LB broth medium with appropriate antibiotic. Plasmid isolation was performed using Promega midiprep kit per the manufacturer's instructions.

2.2.4 Site-directed mutagenesis of plasmids

All molecular biology procedures complied with those outlined in Sambrook and Maniatis (Sambrook., 1989). Site-directed mutagenesis was performed as directed by the two-primer pair method QuickChange™ Site-Directed Mutagenesis Kit by Stratagene (Edinburgh, Scotland). The fidelity of the PCR reaction and subcloning were confirmed by DNA sequencing by Macrogen Inc. (Seoul, Korea).

2.2.5 Plasmid DNA restriction digest and DNA gel electrophoresis

Following NEB guidelines (<http://www.neb.com/nebecomm/default.asp>) the following reagents were added to an Eppendorf tube: 500ng plasmid DNA, 2µl 10x NEBuffer, 0.5µl 100x BSA (if required), 0.5µl restriction enzyme and deionized H₂O to make the final volume 20µl. Mixed and incubated at 37°C for 3h. Samples were then mixed with 5µl DNA running dye (10mM EDTA, 0.25% Bromophenol Blue, 50% Glycerol). Samples were loaded onto a 1% Agarose gel containing gold stain sampler at 1:10,000 dilution so that DNA could be visualised. DNA was separated with an applied potential difference of 100V for about 1h. Gel was observed by illuminating on a 302 nm UV transilluminator. Commercially obtained DNA ladder (Promega) was used as molecular weight marker.

2.2.6 Cell line Culture

All the cell lines were cultured in class II biohazard safety cabinet at 37°C under 5% CO₂ atmosphere. Cells were maintained in the appropriate size tissue culture treated flasks with specific media and growth supplements under adherent conditions. For passaging, cells were washed twice with incomplete media and detached from the tissue culture flask by incubating at 37°C for 1min in 0.25% trypsin/EDTA solution. Trypsinisation was followed by, neutralisation of trypsin with an equal volume of serum supplemented medium. Cells were then recovered by centrifugation at 500rpm for 2min at room temperature. Cell pellet was washed with incomplete media and centrifuged again at 500rpm for 2min at 4°C before re-suspending in appropriate volume of cell growth specific medium.

2.2.7 Cell Viability

Trypan blue is a diazo dye with negatively charged chromophore, which does not interact with the cell until its membrane is damaged. Dead cells thus acquire the dye and appear blue under the microscope while the live cells are excluded from the dye. Cells were detached from tissue culture flask using 0.25% trypsin/EDTA solution followed by neutralisation of trypsin using equal volume of serum containing media. Cell suspension was then mixed with trypan blue solution in 1:9 ratio. Then 10µl of the cell/trypan blue mix was placed on the hemocytometer (Hausser Scientific, PA, USA) to perform cell count under the microscope.

Fluorescent chemical compound propidium iodide (PI) was used to check the cell viability using flow cytometry. Cells were detached from the tissue culture flask using 0.25% trypsin/EDTA solution, followed by neutralisation of trypsin using equal volume of serum containing media. Cell suspension was then washed with PBS, followed by centrifugation at 1000rpm for 5min at 4°C. Cells pellet was re-suspended in 200µl of

PBS supplemented with 0.25µg of PI per million cells, for 5min at 4°C, in dark. Cells were analysed for viability using Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ), and Accuri C6 software.

2.2.8 Calcium phosphate transfection of HEK293T cells

Transfection was carried out on sub confluent HEK293T cell cultures using the calcium phosphate precipitation method. For a 10cm dish with 10ml of culture media, 2.5µg of each plasmid was added to 62µl 2M CaCl₂ with up to 438µl of sterile water. This solution was added drop-wise over the course of two minutes to 500µl hanks buffered salt solution (HBSS) and the resulting cloudy mixture was allowed to stand for three minutes. The 1ml precipitate solution was then added to the cell culture. Essentially the same procedure was performed for each well of a 6-well plate except 200µl of the precipitate solution containing 2µg of total DNA was added per well. In all instances the total amount of DNA in each culture was kept constant by addition of empty vector pcDNA3 (Invitrogen). Media was changed 8-16h post-transfection and cells were harvested 24-48h post-transfection.

2.2.9 Transfection of mouse embryonic fibroblasts (MEFs)

Transfection was carried out on subconfluent MEF cell cultures using the TurboFect™ in vitro transfection reagent from BioRad Laboratories (GmbH, Munich, Germany). Plasmid and TurboFect™ were added in 1:3 ratio and incubated for 5min. 200µl of serum-free DMEM was then added to the above mixture and incubated for 15min at room temperature. Then the combined solution was added drop-wise to the cell culture plates. Media was changed 8-12h post-transfection, and cells were harvested 36-48h post-transfection.

2.2.10 Preparation of cellular protein extracts

HEK293T cell cultures were washed with ice-cold phosphate-buffered saline (PBS) and lysed in 200µl/ml of lysis buffer (50 mM HEPES, 150mM NaCl, 1 mM EDTA, 0.1% (v/v) Nonidet P-40, 1mM sodium orthovanadate and protease inhibitor mixture) for 6 well plates or 10 cm dishes respectively. Cells were lysed on ice for one hour, spun at 13000rpm at 4°C for 10min and supernatants were collected. Lysates were normalised using the bicinchoninic acid (BCA) method by Pierce.

MEF cell cultures were transferred on ice and cell cultures were washed with ice-cold PBS. Cells were scraped in PBS and pellet at 1000rpm at 4°C for 10min. Removing any excess PBS, cells were lysed in cell lysis buffer containing 1X complete protease inhibitor, for 1h. Cells were then syringe and needle lysed to be broken up into viscous lysate. Lysate was spun down at 13000rpm at 4°C for 10min and supernatants were collected. Lysate was normalised to ensure an equal amount of protein loaded to each sample using the bicinchoninic acid (BCA) method by Pierce biotechnology.

2.2.11 Preparation of poly-L-lysine coated plates

Coverslips were washed with 1XPBS and etched with 50% HCl (v/v) for 1h at 60°C. Post etching coverslips were washed three times with 1X PBS and placed in tissue culture plates. Then Poly-L-lysine (Sigma) 1mg/ml stock (1000µg/ml) was diluted down (with sterile-filtered water) to either 100µg/ml (1/10 dilution) or 200µg/ml (1/5 dilution). Next 2ml of diluted poly-L-lysine was added to cover the coverslips for 1h at room temperature. Excess poly-L-lysine was collected and was re-used up to 3 times. Plates were washed three times with sterile-filtered water. Finally plates were allowed to dry in hood over-night or for several hours after which they were used to seed cells.

2.2.12 Immunoprecipitation of proteins from cellular extracts

Equivalent concentrations of lysates (for exogenously expressed proteins 200-500µg of protein lysate and for endogenously expressed proteins 1000µg of protein lysate) were pre-cleared for 1h at 4°C with 25µl Protein-G sepharose beads. Pre-cleared lysates were immunoprecipitated for 2h at 4°C with 2-5µg of the indicated antibody followed by incubation with 25µl Protein-G sepharose beads overnight. Immunoprecipitates were then washed three times in 500mM NaCl lysis buffer followed by two washes in 150mM NaCl lysis buffer. Samples were resolved by 10% or 12% SDS-PAGE, transferred to nitrocellulose membrane and visualised as outlined in 2.2.13.

2.2.13 Immunoprecipitation of proteins from cellular extracts for ubiquitination assay

Cell cultures were washed twice in ice-cold PBS and detached from plates by gentle scraping in 1ml PBS-EDTA (0.5mM). The resulting suspensions were transferred into 1.5ml eppendorf and a sample removed for lysis and western blot analysis. The rest of the cells were harvested by spinning at 1000rpm for 5min at 4°C. Pellets were resuspended in 250µl 1% (w/v) sodium dodecyl sulphate (SDS) containing 15mM N-ethylmaleimide (NEM) and protease inhibitors and boiled for 5min on a heating block. Following cooling on ice, an equal volume of ice-cold covalent buffer (50mM Tris (pH 8.0), 150mM NaCl, 1% Triton, 0.5% sodium deoxycholate, 15 mM NEM and protease inhibitors) was added, lysate was mixed and spun at top speed at 4°C for 20min. The pellet was removed and lysates quantified using the BCA method as previously described. Samples were then subjected to immunoprecipitation with indicated antibodies as described previously, except that Protein-G sepharose beads were washed three times in covalent buffer.

2.2.14 Western blotting

Equivalent concentrations of lysates were denatured by addition of SDS loading buffer and boiling for 5min on a heating block. However when blotting for PS1 or PS2, the boiling step was omitted as boiling PS1 or PS2 disrupts the epitope-antibody recognition site. Similarly, washed Protein G beads were boiled in SDS loading buffer and boiled for 5min on a heating block. Samples were resolved on 10% (w/v) or 12% (w/v) SDS-PAGE gels, transferred to nitrocellulose membrane (Schleicher and Schuell Bioscience). Following transfer, membranes were blocked for 1h at room temperature in 5% (w/v) marvel milk/PBS-Tween 0.1% (v/v). Primary antibodies were diluted as outlined in Appendix 5, either in 5% (w/v) milk or 3% BSA in TBS-Tween 0.1% (v/v) and incubated shaking at 4°C overnight. Membranes were washed three times in TBS-Tween for 10min each and incubated in diluted secondary antibody for 1h at room temperature. Following washing, proteins were detected with the Licor Odyssey Infrared Imaging System for Licor antibodies; ECL illumination kit was used for HRP conjugated antibodies.

2.2.15 ELISA for sTNFR1

HEK293T cells were transfected by calcium phosphate precipitation with indicated constructs. Thirty-six hours after transfection, cell culture medium was collected for ELISA analysis and cells were subjected to western blotting. An ELISA kit (Invitrogen) for sTNFR1 was purchased from Invitrogen. ELISA analyses were carried out according to the manufacturer's protocol.

2.2.16 Flow Cytometry

Cells growing in the tissue culture plates were washed twice with ice cold PBS before detaching with PBS/10 μ M EDTA solution. Cells were re-suspended in 5ml of PBS with BSA (PBSA), and centrifuged at 1000rpm for 5min at 4°C. For extracellular antigens,

cells were re-suspended in 100µl of primary antibody diluted with PBSA, and incubated for 1h on ice. Cells were washed with 1ml of PBSA followed by centrifugation at 1000rpm, 4°C for 5min. 100µl of secondary antibody diluted with PBSA was added, and incubated 30min on ice in dark. Cells were washed twice with 1ml of PBSA, and centrifuged at 1000rpm, 4°C for 5min. Cells were then re suspended in 200-500µl of 1% paraformaldehyde (PFA) for analysis.

2.2.17 Immunostaining for cultured cells

For immunostaining, cells were cultured in 6-well plates on coverslips coated with poly-L-lysine, under specific growth conditions.

Extracellular antigen staining

For extracellular antigens, cells were treated with 100µl of primary antibody diluted with PBSA, and incubated for 1h on ice. After washing twice with 1ml of PBSA, 100µl of secondary antibody diluted with PBSA was added. Cells were incubated for 30min on ice, and washed twice with 1ml of PBSA. Nuclei were counterstained with 1:1000 diluted DAPI/PBS for 20min at room temperature, in dark. Coverslips were mounted on glass slides using prolong gold antifade (Invitrogen). The images were recorded by confocal microscope (Olympus Fluoview confocal microscope system) and analysed using Olympus Fluoview 6 software

Intracellular antigen staining

For cytoplasmic antigens, PFA fixed cells were permeabilized with 0.2% Triton X-100 supplemented with 0.5% BSA with or without 5% normal goat serum for 30min at 4°C. 100µl of primary antibody diluted with supplemented 0.2% Triton X-100 was added to the cells, and incubated for 2h on ice. Cells were washed with 1ml of 0.2% Triton X-100, followed by 60min of incubation with 100µl of secondary antibodies diluted with

supplemented 0.2% Triton X-100, on ice in dark. Cells were washed twice with 1ml of supplemented 0.2% Triton X-100. Nuclei were counterstained with 1:1000 diluted DAPI/PBS for 20min at room temperature in dark. Mounting and imaging of the samples was performed as described in extracellular antigen staining section.

Nuclear antigen staining

For nuclear antigens, cells were permeabilized with 100% methanol at -20°C for 10min, followed by blocking in 0.2% Triton X-100 supplemented with 0.5% BSA with or without 5% normal goat serum for 30min at 4°C. 100µl of primary antibodies diluted with supplemented 0.2% Triton X-100 was added, and incubated for 2h on ice. Cells were washed with 1ml of supplemented 0.2% Triton X-100, followed by incubation with 100µl secondary antibodies diluted with supplemented 0.2% Triton X-100, for 60min on ice in dark. Cells were washed twice with 1ml supplemented 0.2% Triton X-100. Nuclei were counterstained with 1:1000 diluted DAPI/PBS for 20min at room temperature in dark. Mounting and imaging of the samples was performed as described in extracellular antigen staining section.

2.2.18 Subcellular fractionation

After the indicated treatments, cells were washed with ice cold PBS and scraped in 1ml of PBS. The cells were pelleted at 1000rpm at 4°C for 5min. Cell pellet was lysed in 1ml of buffer I and homogenized by passage through a 16.5 gauge needle. Nuclei were spun down at 3000rpm for 10min. The resulting supernatant obtained containing plasma membrane and cytoplasmic fraction. It was either subject to sucrose gradient fractionation to isolate early and late endosome or isolate cytosolic, nucleic and plasma membrane fraction as described below.

Sucrose gradient fractionation

The supernatant obtained above was laid at the bottom of an ultracentrifuge tube. To form a discontinuous sucrose gradient, lysis buffer containing 0.25M (900μl), 0.5M (500μl), 0.8M (300μl), 1.16M (1500μl), 1.3M (1500μl), and 2M (300μl) sucrose were sequentially laid over the homogenate. The samples were ultracentrifuged at 100,000g in a Beckman table top ultracentrifuge swinging rotor SW55 at 4°C for 14-16h. Fifteen 0.3ml fractions were collected from top to bottom of the gradient sample. Equal volumes (48μl) of each fraction and 12μl of 5X sample buffer were subjected to western blot analysis. These immunoblot was then probed with antibodies for each organelle marker- plasma membrane (Na^+/K^+ ATPase), cytosol (β -tubulin/GAPDH), early endosome (EEA1), late endosome (LAMP1), trans-golgi network (TGN-38), nucleus (Histone 2b) and endoplasmic reticulum (PDI). The fractions for early endosome were restricted to fraction 6, 7, 8 and late endosome fraction to 10, 11, and 12.

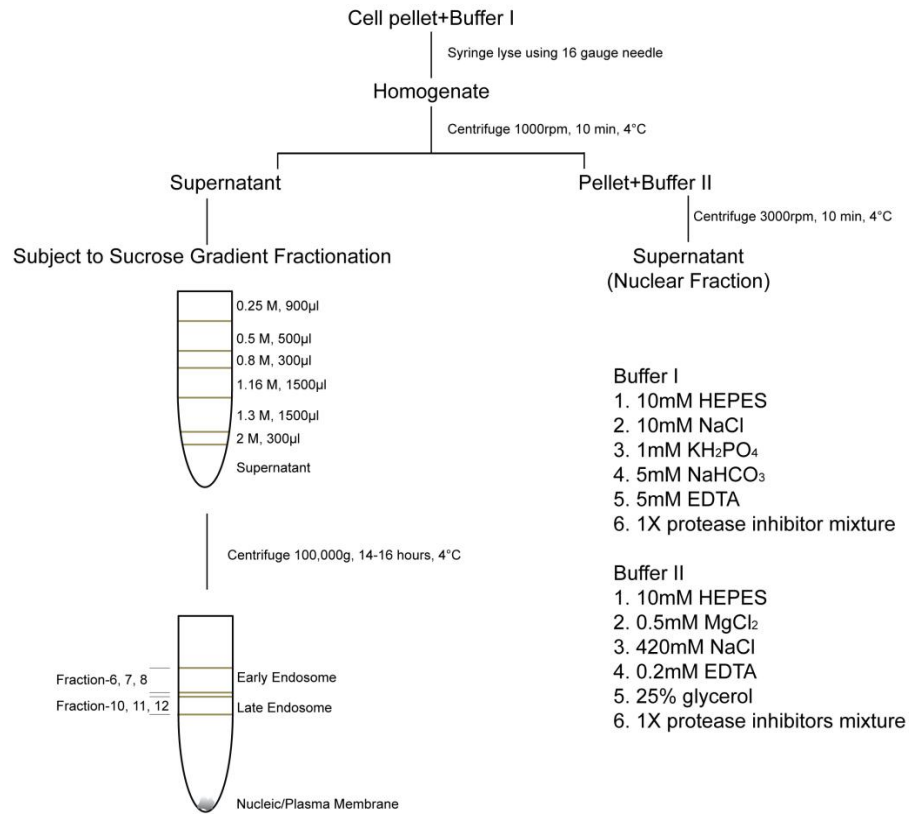
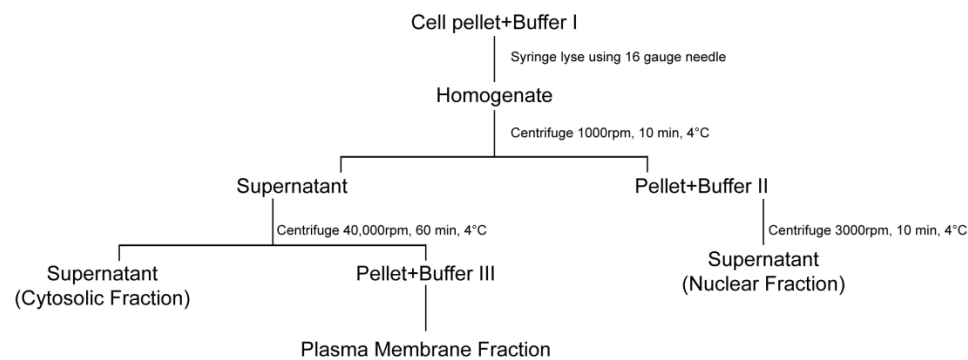


Figure 2 1 Sucrose gradient fractionation to isolate early and late endosome

Cytosolic, nucleic and plasma membrane fractionation

After the indicated treatments, cells were washed with ice cold PBS and lysed in buffer I and homogenized by passage through a 16.5 gauge needle. Nuclei were spun down at 3000rpm for 10min, and the resulting supernatant was then microfuged at 40,000rpm for 60min at 4°C. The supernatant was removed (cytosol), and buffer containing 1% Triton X-100 was added to the pellet (membrane). Nuclear proteins were extracted in buffer II.



Buffer I
1. 10mM HEPES
2. 10mM NaCl
3. 1mM KH₂PO₄
4. 5mM NaHCO₃
5. 5mM EDTA
6. 1X protease inhibitor mixture

Buffer II
1. 10mM HEPES
2. 0.5mM MgCl₂
3. 420mM NaCl
4. 0.2mM EDTA
5. 25% glycerol
6. 1X protease inhibitors mixture

Buffer III
1% Triton X-100

Figure 2 2 Nucleic, cytosolic and plasma membrane fractionation protocol

2.2.19 Statistical Analysis

Quantitative data are shown as mean \pm SEM from three independent experiments unless indicated otherwise. Statistical analysis was performed using GraphPad PRISM 5.0 (GraphPad Software, La Jolla, CA). A value of $p < 0.05$ was considered statistically significant. [* , $p < 0.05$; ** , $p < 0.01$; ***, $p < 0.001$]

Chapter 3

Subcellular localization of the ectodomain shedding and γ -secretase-mediated intramembrane proteolysis of IL-1R1

3.1 Introduction

The γ -secretase protease complexes and associated regulated intramembrane proteolysis play an important role in controlling receptor-mediated intracellular signalling events, which have a central role in AD, cancer progression and immune surveillance. Previously our group reported that IL-1R1 undergoes PMA-induced metalloprotease-mediated ectodomain shedding followed by γ -secretase-mediated regulated intramembrane proteolysis. Furthermore we showed that defective γ -secretase activity alters IL-1 β -mediated IL-6 production (Elzinga et al., 2009). Additionally, using ADAM10 and ADAM17 specific inhibitors, and embryonic fibroblasts from ADAM10^{-/-} and ADAM17^{-/-} knockout mouse, we demonstrated that ADAM17 mediates the ectodomain cleavage event of IL-1R1 (unpublished).

ADAM17 was initially characterized as the TNF α converting enzyme (TACE) and has over 70 substrates (Dreymueller et al., 2012; Moss et al., 1997). ADAM17^{-/-} mice die shortly after birth with open eyelids and defects in epithelial maturation of multiple organs, along with several skin and hair abnormalities (Peschon, 1998). A number of ADAM17 substrates are anti- or pro- inflammatory receptors including: TNFR1, TNFR2, IL1-R2, CD40, and CD163 (Edwards et al., 2008; Weber et al., 2012). Various groups studying the ADAMs have been targeting ADAM17, along with other ADAM family members, using pharmacological inhibitors and gene knock-out approaches to attenuate inflammatory responses in animal models (Dreymueller et al., 2012). The biggest limitation of this approach has been the promiscuity of ADAMs with respect to their substrates, where a single ADAM can target multiple proteins (Crowe et al., 1995; Mullberg et al., 1995; Bennett TA et al., 1996; Coodly, 1996; Moss et al., 1997; Buxbaum, 1998), as well as a single protein can be targeted by multiple ADAMs (Dreymueller et al., 2012). Thus, it is important to study the specific subcellular localization of these cleavage events and the basis of their substrate specificities to prevent the undesirable effects of using ADAM inhibitors in a physiological system.

IL-1R1 belongs to the IL-1R superfamily members defined by their common homologous cytoplasmic TIR domain and the repeated extracellular Ig domains. Upon IL-1 β binding, IL-1R1 associates with IL-1RAcP, which leads to the recruitment of the adaptor proteins and the activation of downstream signalling cascades (Subramaniam et al., 2004). Although many studies have been carried out to understand the functions of IL-1R1 signalling complexes and the exact role played by the adaptor proteins, the subcellular occurrence and recruitment of these adaptor proteins remains elusive. Amongst the IL-1R1 adaptor proteins, TRAF6 has been shown to indirectly regulate a number of IL-1 superfamily members. For instance, TRAF6 is recruited to the IL-1 receptor complex, via its interaction with MyD88 or IRAK leading to NF- κ B activation. Its role is also highlighted by the studies carried out in TRAF6^{-/-} mice which are defective in IL-1-mediated signalling (Lomaga et al., 1999).

A role for TRAF6 in regulated intramembrane proteolysis of IL-1R1 is also highlighted as TRAF6-mediated ubiquitination positively modulates regulated intramembrane proteolysis of IL-1R1 (Twomey et al., 2009). The role played by TRAF6 in IL-1R1 signalling is well characterized, however the role played by TRAF6-mediated ubiquitination of IL-1R1 is not known. In this regard, the involvement of TRAF6 in mediating the ubiquitination of IL-1R1 and its regulated intramembrane proteolysis, co-relates the post-translational modification of IL-1R1 with the adaptor proteins involved in the signalling cascade. This is supported by the observation that IL-1R1 undergoes monoubiquitination upon IL-1 β stimulation, promoting its internalization which is subsequently poly-ubiquitinated and interacts with two ubiquitin-binding proteins, Tollip and Tom1, which modulate sorting of IL-1R1 from late endosome to lysosome for degradation (Brissoni et al., 2006). Thus, indicating the role of IL-1R1 ubiquitination in mediating trafficking and signalling events. We hypothesize that TRAF6-mediated ubiquitination could play an important role in co-relating trafficking, signalling and regulated intramembrane proteolysis of IL-1R1.

Ectodomain shedding and regulated intramembrane proteolysis is tightly regulated cleavage events. Recent studies reflect their importance on trafficking and degradation of substrates thus regulating the signalling cascades. However nothing is known about the subcellular location for ectodomain shedding and regulated intramembrane proteolysis of IL-1R1 or its regulation.

The aims of the research presented in this chapter are-

1. To determine the subcellular location of ectodomain shedding and γ -secretase-mediated cleavage of IL-1R1.
2. To study the role played by TRAF6-mediated ubiquitination of IL-1R1 in regulated intramembrane proteolysis.
3. To determine the fate of the IL-1R1 ICD generated following γ -secretase cleavage.
4. Given that, IL-1R1, IL-1R2, and IL-6 are all characterized substrates for γ -secretase-mediated regulated intramembrane proteolysis, we determined whether or not other members of the IL-1/TLR superfamily were γ -secretase substrates.

3.2 Results

3.2.1 IL-1R1 is a substrate for γ -secretase-mediated proteolysis

To examine the expression of IL-1R1 and verify that it undergoes PMA-induced ectodomain shedding and subsequent γ -secretase-mediated cleavage, the broad spectrum pharmacological inhibitor of γ -secretase, was used. It is a non-competitive, non-transition state analogue inhibitor which overlaps with the active site of γ -secretase (Kornilova et al., 2003). HEK293T cells were transiently transfected with IL-1R1 and forty hours post-transfection cells were pre-treated with DAPT (10 μ m/ml; 8h) or DMSO as a vehicle control in serum-free media. Cells were subsequently treated with PMA (200ng/ml; 2h), alone or in combination with DAPT. The cleavage profile of IL-1R1 was examined by immunoblotting with C-terminus specific anti-IL-1R1 antibody (IL-1R1 C20). Immunoblot analysis revealed that IL-1R1 undergoes constitutive and PMA-induced proteolytic cleavage as evident from the detection of two proteolytic fragments, which based on previous data (Elzinga et al., 2009) correspond to IL-1R1 CTF and IL-1R1 ICD (**Figure 3.1 lane 1 & 2**). The γ -secretase inhibitor, DAPT, completely suppresses the formation of IL-1R1 ICD, (**Figure 3.1 lane 3 & 4**), suggesting that it is indeed the product of γ -secretase-mediated cleavage of IL1-R1 CTF, a parallel increased accumulation of IL-1R1 CTF is also observed. Thus, confirming the expression of the exogenous IL-1R1 and its responsiveness to PMA and γ -secretase protease inhibitor in the cell system being used.

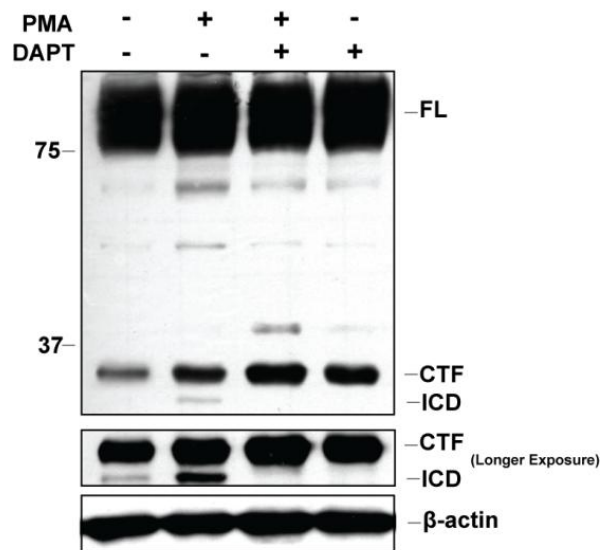


Figure 3.1 IL-R1 is a substrate γ -secretase-mediated cleavage

Immunoblot of cell lysates from HEK293T cells transiently transfected with IL-1R1. Cells were pre-treated with the γ -secretase inhibitor DAPT (10mM/ml; 8h) alone or in combination with PMA (200ng/ml; 2h) as indicated in the figure. C-terminus specific anti-IL-1R1 C20 antibody was used to assess cleavage profile of the receptor. The data is representative of three independent experiments.

3.2.2 Ectodomain shedding is a pre-requisite for γ -secretase-mediated generation of IL-1R1 ICD

All the known γ -secretase substrates have been shown to undergo ectodomain shedding as a pre-requisite to γ -secretase-mediated cleavage (Haapasalo et al., 2012). Next, using a TACE specific hydroxamate based metalloprotease inhibitor, TAPI, we studied the importance of IL-1R1 ectodomain shedding in its γ -secretase-mediated cleavage.

HEK293T cells were transiently transfected with IL-1R1 and forty hours post-transfection cells were pre-treated with TAPI, (50 μ M/ml; 2h) in serum-free media, and subsequently treated with PMA (200ng/ml; 2h) to induce ectodomain shedding. Immunoblot analysis of IL-1R1 cleavage profile in untreated and PMA-treated cells revealed that TAPI inhibits both constitutive and PMA-induced ectodomain shedding and formation of the IL-1R1 CTF (**Figure 3.2 compare lane 1, 2 with lane 3, 4 respectively**), and a simultaneous reduction in the generation of IL-1R1 ICD is also observed (**Figure 3.2 lane 3**). This data suggests that ectodomain shedding is a pre-requisite for γ -secretase-mediated cleavage of IL-1R1.

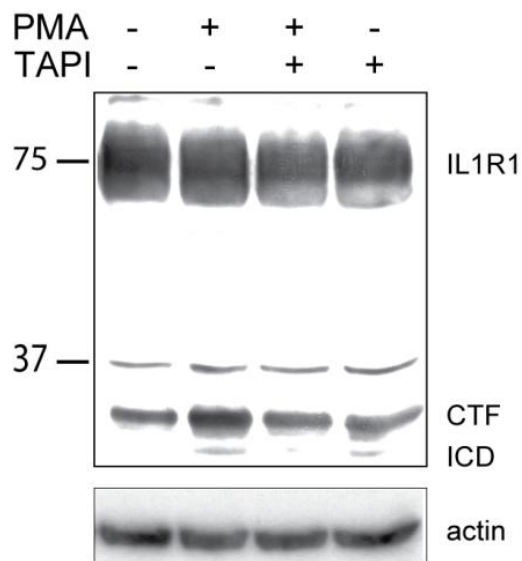


Figure 3.2 IL-1R1 ectodomain shedding is a pre-requisite for γ -secretase-mediated cleavage

Immunoblot of cell lysates from HEK293T cells transiently transfected with IL-1R1 and pre-treated with the broad spectrum metalloprotease inhibitor TAPI (50 μ M/ml; 2h), alone or in combination with PMA (200ng/ml; 2h) as indicated. C-terminus specific anti-IL-1R1 C20 antibody was used to assess cleavage profile of the receptor. The data is representative of three independent experiments.

3.2.3 IL-1R1 internalization is required for IL-1 β and PMA-induced ectodomain shedding

Having shown that ectodomain shedding precedes cleavage by γ -secretase protease complex, we next wanted to determine the subcellular location for this cleavage event. Following ligand binding, IL-1R1 undergoes clathrin-mediated internalization, which is critical for IL-1 induced gene expression (Hansen et al., 2013). Thus, using both pharmacological and genetic approaches, IL-1R1 internalization was inhibited to determine whether ectodomain shedding of IL-1R1 occurs on the cell surface or post-receptor-internalization. Dynamin is a small GTPase required for the delivery of proteins from the clathrin- and caveolin-coated vesicles formed at the plasma membrane to early endosomes (Hinshaw., 2002). In this context, dynasore a noncompetitive inhibitor of dynamin GTPase activity that blocks dynamin-dependent endocytosis (Macia et al., 2006) and dynamin K44A mutant (Dyn-DN) with low affinity for guanylnucleotides and defective in receptor internalization (E. Lee et al., 2001) were used in our studies.

3.2.3.1 Pharmacological inhibition of internalization

HEK293T cells were transiently transfected with IL-1R1 and forty hours post-transfection, cells were pre-treated with dynasore (50 μ M/ml; 2h) or DMSO as a vehicle control in serum-free media followed by IL-1 β (50ng/ml; 1h or 2h) treatment. Immunoblot analysis revealed both constitutive and IL-1 β induced increase in IL-1R1 CTF formation (**Figure 3.3 lane 2 & 4**), which is inhibited in the presence of dynasore (**Figure 3.3 lane 3 & 5**). To verify that dynasore is indeed inhibiting IL-1R1 internalization, RAW264.7 cells were analyzed for IL-1 β induced internalization of IL-1R1. RAW264.7 cells were grown on coverslips up till 70% confluency following which cells were pre-treated with dynasore (50 μ M; 2h) or DMSO as a vehicle control in

serum-free media. Subsequently cells were stimulated with IL-1 β (30ng/ml; 1h or 3h) and stained for either cell surface IL-1R1 or total IL-1R1.

For analysis of cell surface levels of IL-1R1, cells grown on coverslips were stained with an N-terminus specific anti-IL-1R1 antibody (IL-1R1 N20), followed by staining with the anti-rabbit-Alexa Fluor-488; DAPI was used to stain the nucleus. The coverslips were then mounted on a glass slide and analysed using confocal microscopy. To determine the levels of total IL-1R1 in cells, cells grown on coverslips were first fixed with 4% PFA and then permeabilized using 0.1% Triton X-100 in 3% BSA for 2h. Cells were then stained using an N-terminus specific anti-IL-1R1 antibody, followed by staining with anti-Alexa Fluor-488. Samples were imaged using Olympus Fluoview confocal microscope and analysed using Fluoview FV 1000 software. While the cells treated with IL-1 β show decreased levels of cell surface staining for IL-1R1, dynasore treated cells upon stimulation with IL-1 β show no decrease in the cell surface levels of IL-1R1

Figure 3.4.

This data demonstrates that inhibiting IL-1R1 internalization negatively affected TACE-mediated ectodomain shedding of IL-1R1, indicating that IL-1R1 internalization precedes TACE-mediated ectodomain shedding.

a

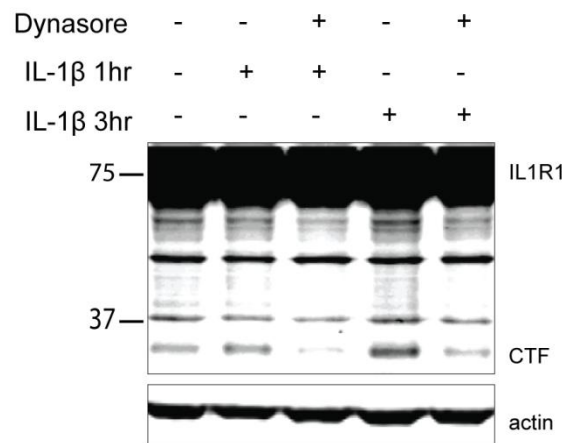


Figure 3.3 Inhibiting internalization inhibits ectodomain shedding of IL-1R1

Immunoblots of cell lysates from HEK293T cells transiently transfected with IL-1R1 and treated as indicated. C-terminus specific anti-IL-1R1 C20 antibody was used to assess cleavage profile of the receptor. The data is representative of three independent experiments.

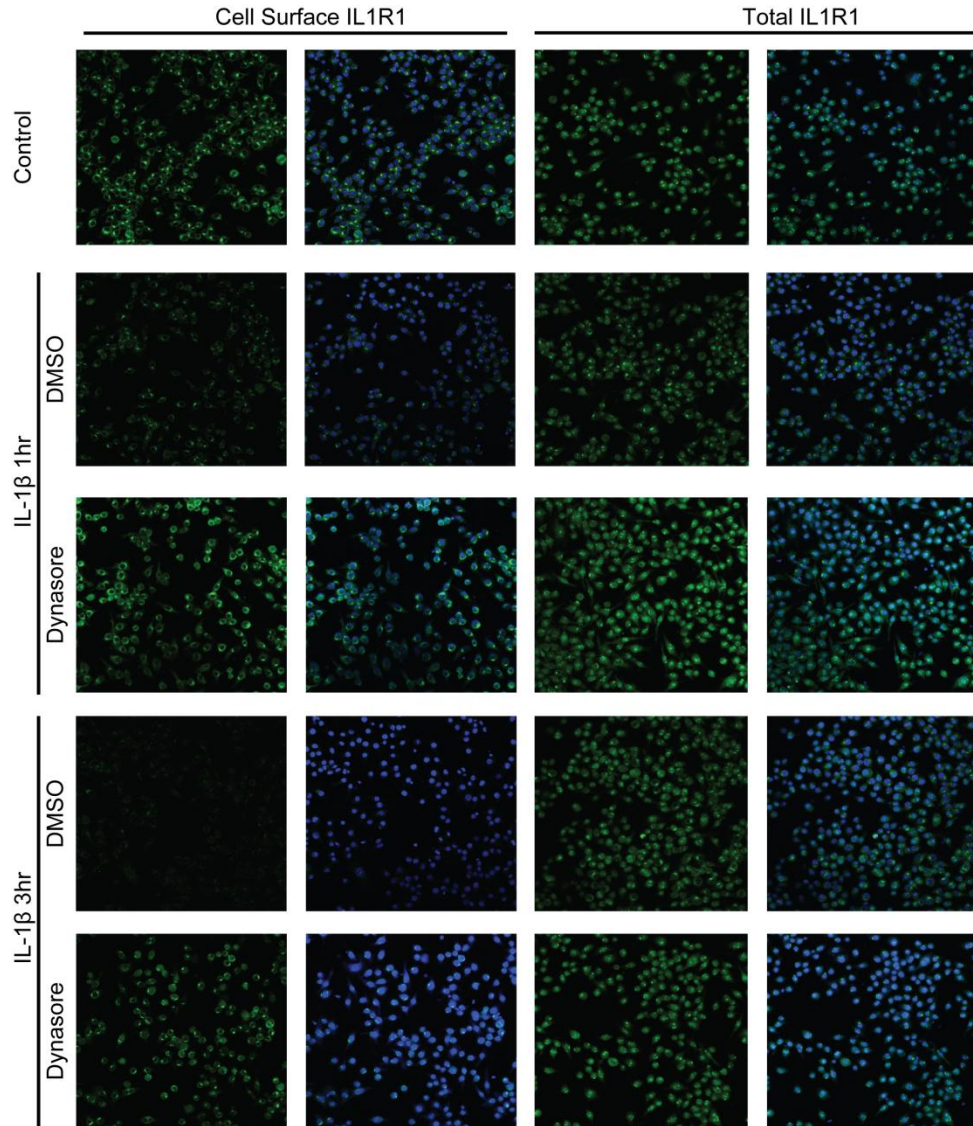


Figure 3.4 Ectodomain shedding is a pre-requisite to γ -secretase-mediated cleavage of IL-1R1

RAW264.7 cells were treated with dynasore alone or dynasore and IL-1 β for indicated time. Cells were then stained for cell surface IL-1R1 or after permeabilization for total IL-1R1 with anti-IL-1R1 N-terminus antibody. Samples were imaged using Olympus fluoview confocal microscope and analysed using Fluoview FV 1000 software. Green: IL-1R1; Blue: DAPI. The data is representative of three separately imaged samples.

3.2.3.2 Dynamin K44A mutant reduces PMA-induced ectodomain shedding of IL-1R1

HEK293T cells were transiently transfected with IL-1R1 and co-transfected with either empty vector or dynamin K44A mutant (Dyn-DN), forty hours post-transfection cells were treated with PMA (200ng/ml) to induce ectodomain shedding of IL-1R1. Immunoblot analysis revealed that while constitutive and PMA-induced formation of IL-1R1 CTF and ICD are seen in cells transfected with IL-1R1 (**Figure 3.5 a lane 1 & 2**), co-expression of Dyn-DN inhibited PMA-induced IL-1R1 CTF formation but did not affect constitutive ectodomain shedding of IL-1R1 and generation of IL-1R1 CTF (**Figure 3.5 compare lane 3 & 4**). To validate the functionality of the Dyn-DN as an inhibitor of IL-1R1 internalization, cell surface levels of IL-1R1 in untreated and PMA treated cells were analysed. HEK293T cells were grown on coverslips and transfected with IL-1R1 and co-transfected with either empty vector or Dyn-DN, and forty hours post-transfection cells were either left untreated or stimulated with PMA (200ng/ml; 2h). Cells were then stained with anti-IL-1R1 N20 antibody followed by anti-rabbit Alexa Fluor-488 antibody; DAPI was used to stain the nucleus. Samples were imaged using Olympus Fluoview confocal microscope and analysed using Fluoview FV 1000 software. Cells treated with PMA show punctate red staining for IL-1R1 in the cytoplasm, suggesting internalization of the receptor as indicated with the arrows (**Figure 3.5 left panels**). In contrast, in cells expressing Dyn-DN, IL-1R1 staining was observed primarily at the cell surface in both untreated and PMA-treated cells, demonstrating that Dyn-DN blocked the internalization of IL-1R1 as indicated with the arrows (**Figure 3.5 right panels**). This data demonstrates that Dyn-DN inhibits PMA-induced ectodomain shedding of IL-1R1.

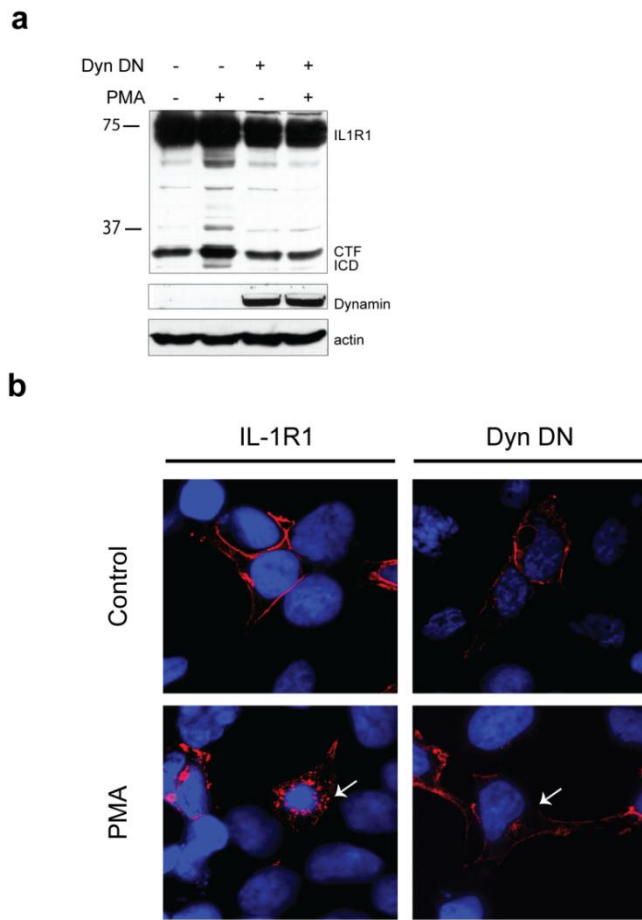


Figure 3.5 Dynamin K44A mutant reduces PMA-induced IL-1R1 ectodomain shedding
(a) Immunoblots of cell lysates from HEK293T cells transiently transfected with IL-1R1 and either empty vector or Dyn-DN. At forty hours post-transfection cells were treated with PMA (200ng/ml; 2h) or DMSO as a vehicle control in serum-free media. **(b)** Cells co-expressing IL-1R1 and empty vector or dynamin DN, were grown on coverslips. At forty hours post-transfection cells were treated with PMA and stained with anti-IL-1R1 antibody followed by anti-rabbit Alexa Fluor-488. Samples were analysed using Olympus fluoview confocal microscope and fluoview FV1000 software. Red: IL-1R1; Blue: DAPI The data is representative of three independent experiments.

3.2.4 IL-1R1 ectodomain shedding occurs in the endosomes

To complement the data obtained from inhibiting clathrin-mediated receptor internalization, the subcellular location of TACE-mediated ectodomain shedding was examined using cells over-expressing Rab5 proteins. Rab proteins belong to a superfamily of small molecular weight GTPases known to be associated with membrane trafficking. Rab5 protein mediates the fusion of endocytic vesicles and trafficking to the early endosomes (Stenmark et al., 2001). Using wild-type (WT), dominant active (DA) and dominant negative (DN) Rab5 mutants, the role of internalization and trafficking was studied for the ectodomain shedding of IL-1R1.

HEK293T cells were transfected with IL-1R1 and co-transfected with Rab5 WT, Rab5 DN or Rab5 DA constructs; forty hours post-transfection cells were treated with PMA (200ng/ml; 2h) in serum-free media to promote ectodomain shedding and formation of IL-1R1 CTF and ICD. Immunoblot analysis revealed that expression of Rab5 DN, inhibited PMA-induced formation of IL-1R1 CTF and IL-1R1 ICD (**Figure 3.6 Figure 3.a compare lane 3 & 4**). On the other hand expression of Rab5 DA led to increased formation of IL-1R1 CTF and IL-1R1 ICD(**Figure 3.6 a compare lane 1 & 2 with lane 5 & 6**). This data shows that, Rab5 DN inhibits PMA-induced IL-1R1 CTF formation while the Rab5 DA, enhances the PMA-induced formation of both IL-1R1 CTF and ICD.

Simultaneous immunofluorescence analysis was done to check the efficacy of the Rab5 DN and Rab5 DA mutants employed in the study. HEK293T cells were grown on coverslips and transfected with IL-1R1 and co-transfected with Rab5 WT, Rab5 DN, or Rab5 DA as indicated in **Figure 3.6 b**; forty hours post-transfection cells were treated with PMA (200ng/ml; 2h) in serum-free media. Cells were then fixed, permeabilized and stained using N-terminus specific anti-IL-1R1 antibody. The immunofluorescence analysis revealed that Rab5 DN prevented PMA-induced trafficking of IL-1R1 to the early endosomes, as seen with corresponding increase in accumulation of the receptor

in the sorting endosomes and a simultaneous increase in the accumulation of the unfused endocytic vesicle (**Figure 3.6**). This is indicated by increased formation of circular vesicles and increased co-staining of IL-1R1 and Rab5 positive vesicles seen in yellow after PMA treatment with no change in cell surface level of IL-1R1 (indicated with the arrows). Co-localization of IL-1R1 and endocytic vesicles was either decreased or absent in cells co-transfected with Rab5 WT or Rab5 DA respectively (indicated with the arrows) (**Figure 3.6b**). This data shows that Rab5 DN prevents trafficking of IL-1R1 from sorting endosomes to early endosomes, thus inhibiting PMA-induced ectodomain shedding and formation of IL-1R1 CTF.

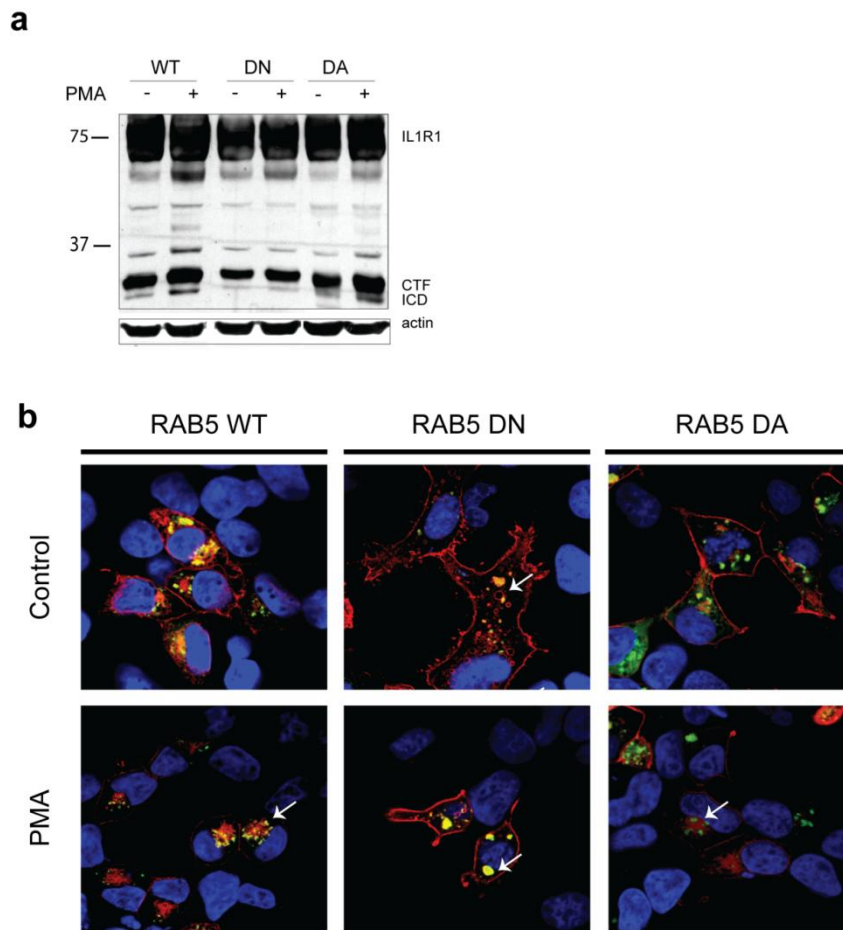


Figure 3.6 IL-1R1 ectodomain shedding occurs in the endosomes

(a) Immunoblot of cell lysates from HEK293T cells transiently transfected with IL-1R1 and either Rab5 WT, Rab5 DN or Rab5 DA. At forty hours post-transfection cells were treated with PMA (200ng/ml; 2h) in serum-free media. Anti-IL-1R1 C20 antibody was used to assess cleavage profile of the receptor. **(b)** Cells grown on coverslips and co-expressing IL-1R1 and either empty vector or Rab5 WT, Rab5 DN or Rab5 DA, were stimulated with PMA and stained with anti-IL-1R1 antibody and stained using Alexa Fluor 594. Samples were analysed using Olympus fluoview confocal microscope and fluoview FV1000 software. Red: IL-1R1; Green: Rab5 positive vesicles; Yellow: Co-localized IL-1R1 and Rab5 vesicles Blue: DAPI The data is representative of three independent experiments.

3.2.5 Mapping the IL-1R1 internalization site

Next, to further characterize the role played by IL-1R1 ectodomain shedding, canonical internalization motifs known to mediate clathrin-dependent receptor internalization were mapped. In this context, four highly conserved tyrosine-based sorting signals [YXX ϕ] were found in IL-1R1 extracellular and the cytoplasmic domain(**Figure 3.7**). The tyrosine residues in the motif are known to be responsible for receptor endocytosis. Two of these motifs were in the extracellular domain, while the other two were present in the ICD. Previous studies have shown that IL-1R1 lacking the cytoplasmic domain still binds to IL-1 β and undergoes internalization, thus showing that the ICD of IL-1R1 is dispensable for its internalization. Therefore, residues W262 and Y278 in the IL-1R1 extracellular domain were mutated to alanine and the mutants were characterized in cell-based assays.

Transferrin receptor	16-PLS Y TR F SLA-35-Tm
Tumor Necrosis Factor Receptor1	Tm-R Y QR W K
Asialoglycoprotein receptor H1	MTKE Y QD L QHL-29-Tm
CD63	Tm-KSIRSG Y EV M
IL-1R1	Tm- 14-C Y DF L I
IL-1R1	Tm- 64- Y KL F I
IL-1R1	SDIA Y WK W -74-Tm
IL-1R1	LGED Y SS V -55-Tm

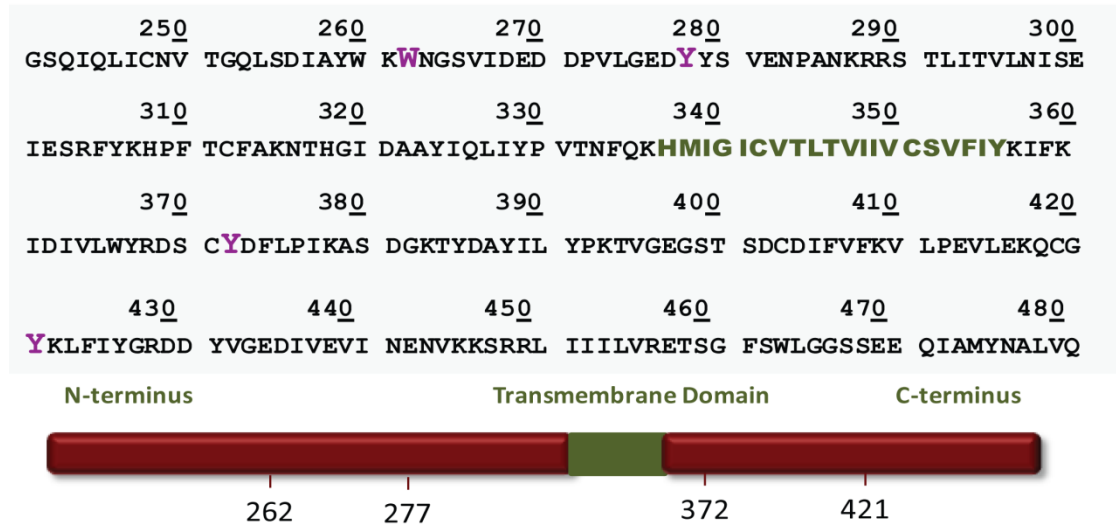


Figure 3.7 Mapping the internalization motif for IL-1R1

(a) Alignment of receptors with known internalization motifs and IL-1R1. **(b)** Schematic representation of the four IL-1R1 amino acids which are tentative residues involved in the internalization of the receptor.

3.2.6 IL-1R1 W262A mutant is defective in ectodomain shedding

HEK293T cells were transfected with wild-type IL-1R1, IL-1R1 W262A or IL-1R1 Y278A mutants and forty hours post-transfection cells were treated with PMA (200ng/ml; 2 h) and processed for western blot analysis. In cells expressing wild-type IL-1R1 or IL-1R1 Y278A mutant, IL-1R1 underwent both constitutive and PMA-induced regulated intramembrane proteolysis, as determined by clear detection of IL-1R1 CTF and ICD (**Figure 3.8a**). In contrast, IL-1R1 W262A mutant was defective in both constitutive and PMA-induced regulated intramembrane proteolysis and abolished formation of both IL-1R1 CTF and IL-1R1 ICD (**Figure 3.8 a lane 3 and 4**). To validate that the IL-1R1 W262A mutant was defective in receptor internalization, cell surface levels of IL-1R1 W262A in untreated and PMA treated cells was analysed. HEK293T cells were grown on coverslips and transfected with either wild-type IL-1R1 or IL-1R1 W262A mutant, and forty hours post-transfection cells were either left untreated or stimulated with PMA (200ng/ml; 2h). Cells were then stained with anti-IL-1R1 N20 antibody followed by anti-rabbit Alexa Fluor-488 antibody; DAPI was used to stain the nucleus. Samples were imaged using Olympus Fluoview confocal microscope and analysed using Fluoview FV 1000 software. Cells treated with PMA show punctate red staining for IL-1R1 in the cytoplasm, suggesting internalization of the receptor (**Figure 3.8 b left panels**). However, in cells expressing IL-1R1 W262A mutant, IL-1R1 staining was only observed at the cell surface in untreated and PMA-treated cells, suggesting that IL-1R1 W262A mutant is defective in internalization (**Figure 3.8b right panels**).

Due to differences between the expression levels of wild-type IL-1R1 and IL-1R1 W262A mutant the immunofluorescence results were not distinctly clear, though it was still evident that the IL-1R1 W262A mutant localized on the cell surface post PMA treatment. Collectively, this data demonstrates that the W262 residue is required for IL-1R1 internalization and subsequent ectodomain shedding.

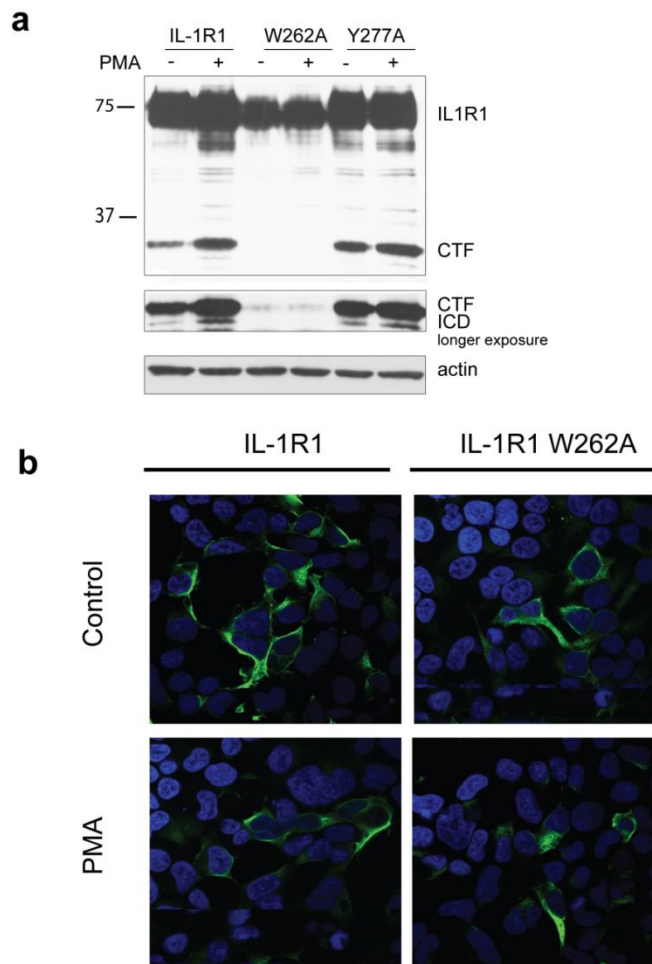


Figure 3.8 IL-1R1 W262A is defective in ectodomain shedding

(a) Immunoblot analysis of whole cell lysates from HEK293T cells transiently transfected with wild-type IL-1R1, IL-1R1 W262A and IL-1R1 Y278A. Blots were analysed using C-terminus specific anti-IL-1R1 antibody. **(b)** Cells expressing wild-type IL-1R1 and IL-1R1 W262A were grown on coverslips. At forty hours post-transfection cells were treated with PMA and stained with anti-IL-1R1 antibody followed by anti-rabbit Alexa Fluor-488. The data represents increased detection of IL1R1 within in the cytosol in PMA treated IL1R1 expressing cells, which is decreased in IL1R1 W262A expressing cells. Samples were analysed using Olympus fluoview confocal microscope and fluoview FV1000 software. Green: IL-1R1; Blue: DAPI The data is representative of three independent experiments.

3.2.7 IL-1R1 ICD undergoes proteasomal degradation

The results so far demonstrates that preventing the trafficking of IL-1R1 to late endosomes, leads to increased formation of the IL-1R1 ICD. However, inhibiting trafficking to late endosome, in turn inhibits subsequent trafficking to the lysosome. Thus, it still remains unclear if increased IL-1R1 ICD formation is attributed to the subsequent defect in trafficking of IL-1R1 from late endosome to lysosome, which in turn inhibits its degradation. Thus, specific inhibitors for both lysosomal and proteasomal degradation were employed in this study. HEK293T cells were transfected with IL-1R1, and forty hours post-transfection, cells were pre-treated with epoximicin: a proteasomal inhibitor (10 μ M/ml; 2h) and concanamycin: a lysosomal inhibitor (50 μ M/ml; 2h) in serum-free media, following which the cells were treated with PMA (200ng/ml; 2h). Increased accumulation of IL-1R1 CTF and ICD in epoximicin treated cells was seen ((**Figure 3.9 lanes 3 & 4**), in contrast concanamycin treatment led to increased accumulation of the IL-1R1 full length and IL-1R1 CTF ((**Figure 3.9 lanes 5 & 6**). This data shows that IL-1R1 ICD undergoes proteasomal degradation.

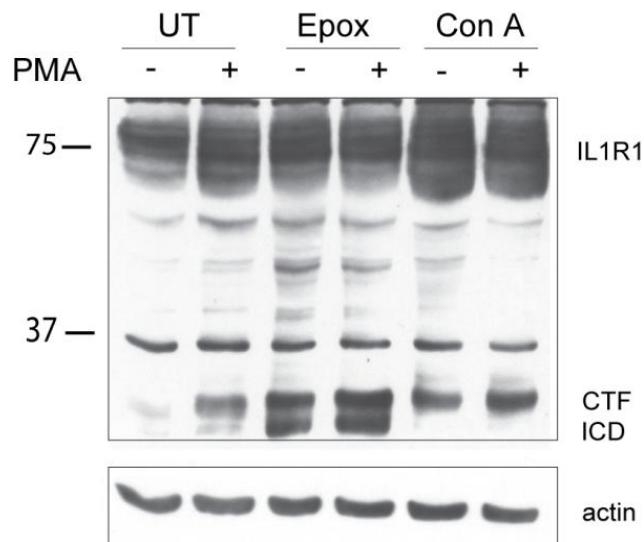


Figure 3.9 IL-1R1 CTF is regulated by both lysosomal and proteasomal degradation pathway; IL-1R1 ICD undergoes proteasomal degradation

Immunoblot of cell lysates from HEK293T cells transiently transfected with IL-1R1 and pre-treated with either epoximycin (10 μ M) or concanamycin A (50 μ M) for 2 hours. Following pre-treatment the cells were treated with PMA (200ng/ml; 2h). C-terminus specific anti-IL-1R1 C20 antibody was used to assess cleavage profile of the receptor. The data is representative of three independent experiments.

3.2.8 TRAF6-mediated ubiquitination of IL-1R1

3.2.8.1 TRAF6-mediated IL-1R1 ubiquitination occurs post-internalization

Previously our group showed that IL-1R1 is a substrate for TRAF6-mediated ubiquitination and also that the E3 ligase activity of TRAF6 enhances regulated intramembrane proteolysis of IL-1R1 (Twomey et al., 2009). Interestingly, TRAF6 is recruited to the IL-1R1 signalling complex in the early endosome (Li et al., 2006), and is required for association of IL-1R1 signalling complex with IRAK1 and also for the recruitment of TAK1 complex by facilitating K63-linked polyubiquitination on the substrates or itself (Cao et al., 1996; Deng et al., 2000; Ninomiya-tsuji et al., 1999). The data obtained so far demonstrates that IL-1R1 ectodomain shedding also occurs on the early endosome. Based on these observations, we studied the mechanism through which TRAF6-mediated ubiquitination of IL-1R1 enhances its regulated intramembrane proteolysis by firstly determining the type of ubiquitination mediated by TRAF6 and additionally looking at whether TRAF6-mediated ubiquitination of IL-1R1 occurs on the cell surface or the endosome i.e., prior to- or post- IL-1R1 ectodomain shedding.

HEK293T cells were transiently transfected with IL-1R1, TRAF6 and co-transfected with HA tagged Ubiquitin (HA-Ub) to study the effect of TRAF6 on IL-1R1 ubiquitination and regulated intramembrane proteolysis. To further characterize the type of ubiquitination mediated by TRAF6, cells were alternatively co-transfected with HA-tagged ubiquitin with lysine residue numbered 63 mutated to arginine (HA-Ub K63R) not capable of undergoing lysine 63 linked polyubiquitination. Additionally to determine the subcellular occurrence of TRAF6-mediated ubiquitination of IL-1R1, Dyn DN which prevents internalization of IL-1R1 was also included in this study. Cells were transfected as indicated (**Figure 3.10**), and forty hours post-transfection cells were

lysed and immunoblotted with respective antibodies to confirm the expression of constructs and equivalency of loading.

After confirming the equivalent expression of the various plasmids, cells were lysed under stringent SDS-denaturing conditions. IL-1R1 was immunoprecipitated with a C-terminus specific anti-IL-1R1 antibody followed by western blotting with anti-HA or anti-IL-1R1 antibody to detect ubiquitinated-IL-1R1 and IL-1R1 respectively. As expected, TRAF6 enhanced regulated intramembrane proteolysis of IL-1R1 demonstrated by increased levels of IL-1R1 CTF and ICD (**Figure 3.10 lane 3 lower panel**). Also, this data demonstrates that IL-1R1 undergoes TRAF6-mediated K63-linked ubiquitination post-internalization, since there was no difference in the ubiquitination pattern of the full length IL-1R1 in presence of internalization inhibitor mutant Dyn-DN and the K63R mutant (**Figure 3.10 lane 4 & 7**). Collectively this data demonstrates TRAF6-mediated K63 linked ubiquitination of IL-1R1 occurs post-internalization.

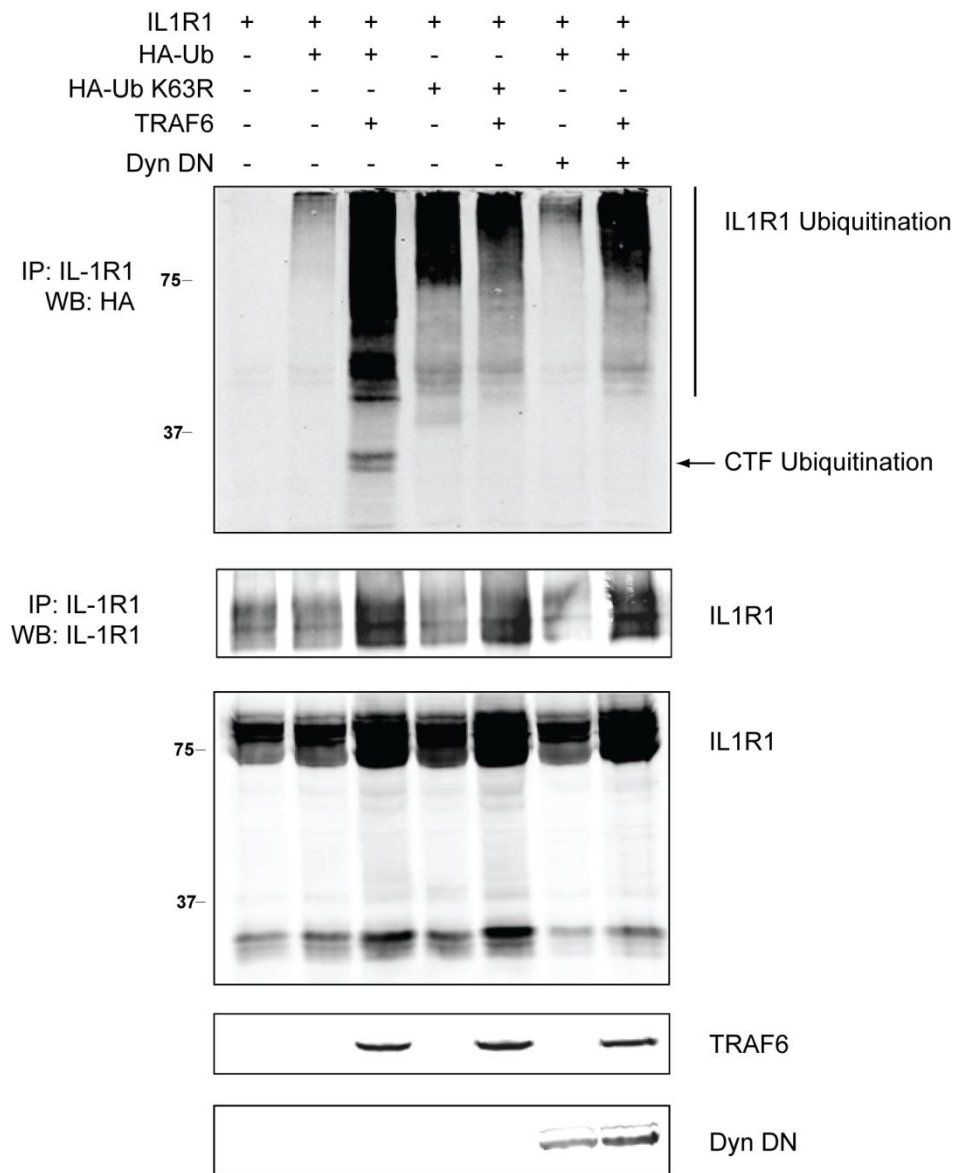


Figure 3.10 TRAF6-mediated ubiquitination of IL-1R1 occurs post-internalization

HEK293T cells were transfected with wild-type IL-1R1 and co-transfected with HA-Ub or HA-Ub K63R and TRAF6 and Dyn K44A. Forty hours post-transfection cells were harvested under stringent SDS denaturing condition and then subjected to immunoprecipitation for IL-1R1 and Western blot for HA-Ub. Ubiquitinated IL-1R1 full-length or CTF were detected with anti-HA antibody. The data is representative of three independent experiments.

3.2.8.2 IL-1R1 K360/378/383R mutant is defective in TRAF6-induced IL-1R1 ectodomain shedding

In an independent study carried out in our group, TRAF6-mediated ubiquitination of IL-1R1 was shown to occur between amino acids 357 and 390 in the cytosolic domain of IL-1R1 (unpublished). There are four lysine residues in IL-1R1 juxtamembrane domain- K356, K360, K378 and K383 (**Figure 3.11**). These residues were mutated making IL-1R1 K360/378R, K357/360/378R and K360/378/380R double or triple lysine mutants. We analysed these mutants for any defect in TRAF6-mediated ubiquitination. HEK293T cells were transiently transfected with IL-1R1 wild-type, IL-1R1 K360/378R, IL-1R1 K357/360/378R or IL-1R1 K360/378/380R, and co-transfected with HA-Ub and either empty vector or TRAF6. At forty hours post-transfection cells were lysed and immunoblotted with respective antibodies to confirm the expression of constructs and equivalency of loading (**Figure 3.11b**). After confirming the equivalent expression of the various plasmids, cells were lysed under stringent SDS-denaturing conditions. IL-1R1 was immunoprecipitated with a C-terminus specific anti-IL-1R1 antibody followed by western blotting with anti-HA or anti-IL-1R1 antibody to detect ubiquitinated-IL-1R1 and IL-1R1 respectively. In cells expressing wild-type IL-1R1, IL-1R1 K360/378R or IL-1R1 K357/360/378R mutant, both constitutive and TRAF6-mediated ubiquitination (**Figure 3.11 upper panels**) and regulated intramembrane proteolysis of IL-1R1 was evident, as IL-1R1 CTF and ICD were clearly detected (**Figure 3.11 lower panels**). In contrast, in cells expressing IL-1R1 K360/378/380R mutant, co-expression of TRAF6 failed to increase IL-1R1 CTF ubiquitination (**Figure 3.11 upper panels**) and regulated intramembrane proteolysis of IL-1R1, and no increase in IL-1R1 CTF was detected (**Figure 3.11 lane 7 & 8**).

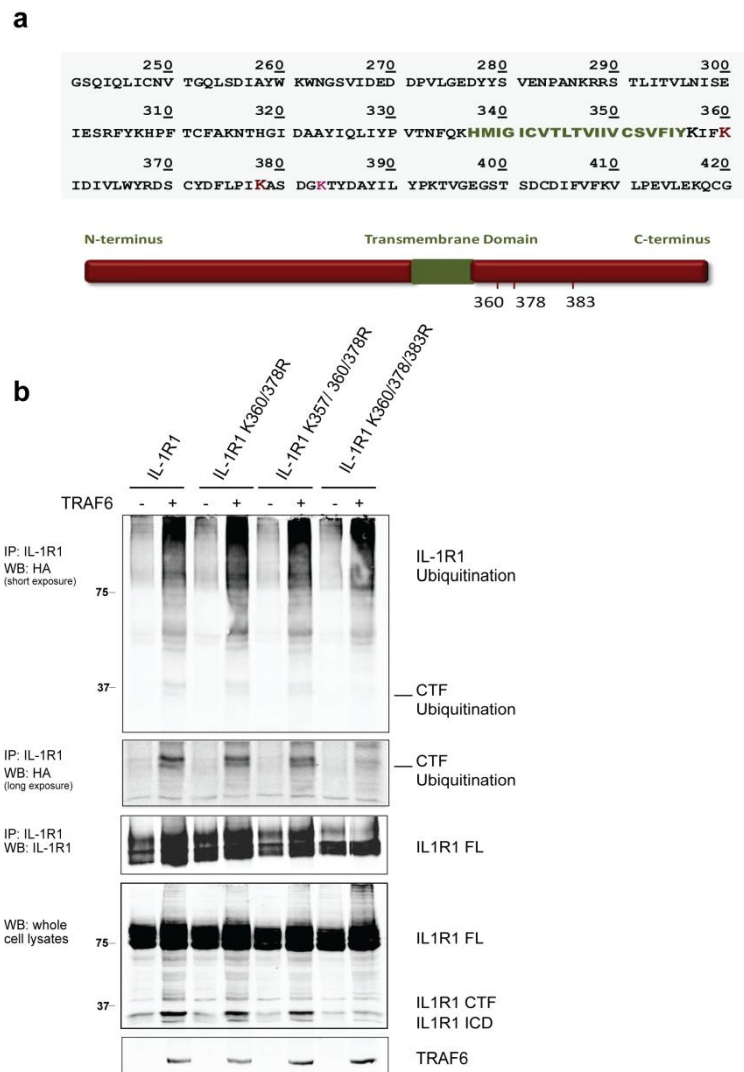


Figure 3.11 IL-1R1 K360/378/383R mutant is defective in TRAF6-induced ectodomain shedding

(a) Schematic representation of juxtamembrane IL-1R1 lysine residues mutated to arginine. **(b)** Immunoblot analysis of HEK293T cells transfected with wild-type IL-1R1, IL-1R1 lysine mutants and co-transfected with TRAF6. Forty hours post-transfection cells were harvested under stringent SDS denaturing condition and then subjected to immunoprecipitation for IL-1R1 and Western blot for HA-Ub. Ubiquitinated IL-1R1 full-length or CTF were detected with anti-HA antibody. The data is representative of two independent experiments.

3.2.8.3 IL-1R1 K360/378/383R mutant is not defective in internalization

Next we assessed the effect of mutagenesis of these three lysine residues on the cell surface localization and its PMA-induced internalization of IL-1R1. HEK293T cells were transfected with empty vector, wild type IL-1R1 or IL-1R1 K360/378/380R mutant; forty hours post-transfection, cells were treated with PMA (200ng/ml; 2h), lysed and analysed by western blot. Immunoblot analysis revealed that while wild-type IL-1R1 showed both constitutive and PMA-induced accumulation of IL-1R1 CTF and ICD formation (**Figure 3.12 a lane 1 & 2**). In contrast, the IL-1R1 K360/378/383R mutant showed decreased levels of constitutively cleaved and PMA-induced formation of IL-1R1 CTF (**Figure 3.12 a compare lane 1 & 2 with lane 3 & 4**). Interestingly, IL-1R1 ICD was still being formed upon PMA-stimulation (**Figure 3.12 a lane 4**).

In parallel cells were grown on coverslips, transfected and treated as above. Post-treatment cells were fixed and probed with anti-IL-1R1 antibody for cell surface staining. PMA treatment induced internalization of the receptor in both wild-type IL-1R1 and IL-1R1 K360/378/383R mutant, as seen by increased number of vesicles indicated by punctate staining within the cell (**Figure 3.12 b**), clearly indicating that the IL-1R1 K3R mutant was not defective in internalization. The cell surface levels of wild-type IL-1R1 and IL-1R1 K360/378/383R mutant were also studied using flow cytometry. HEK293T cells expressing IL-1R1 or IL-1R1 K360/378/383R mutant and treated with PMA (200ng/ml; 2h) were detached and incubated with anti-IL-1R1 antibody for 45 minutes on ice. Following which the cells were incubated with secondary Alexa-Fluor-488 antibody for 30 minutes. Levels of cell surface IL-1R1 were measured by flow cytometry. HEK293T cells over-expressing IL-1R1 and IL-1R1 K360/378/380R mutant showed similar levels of cell surface localization (**Figure 3.12c**).

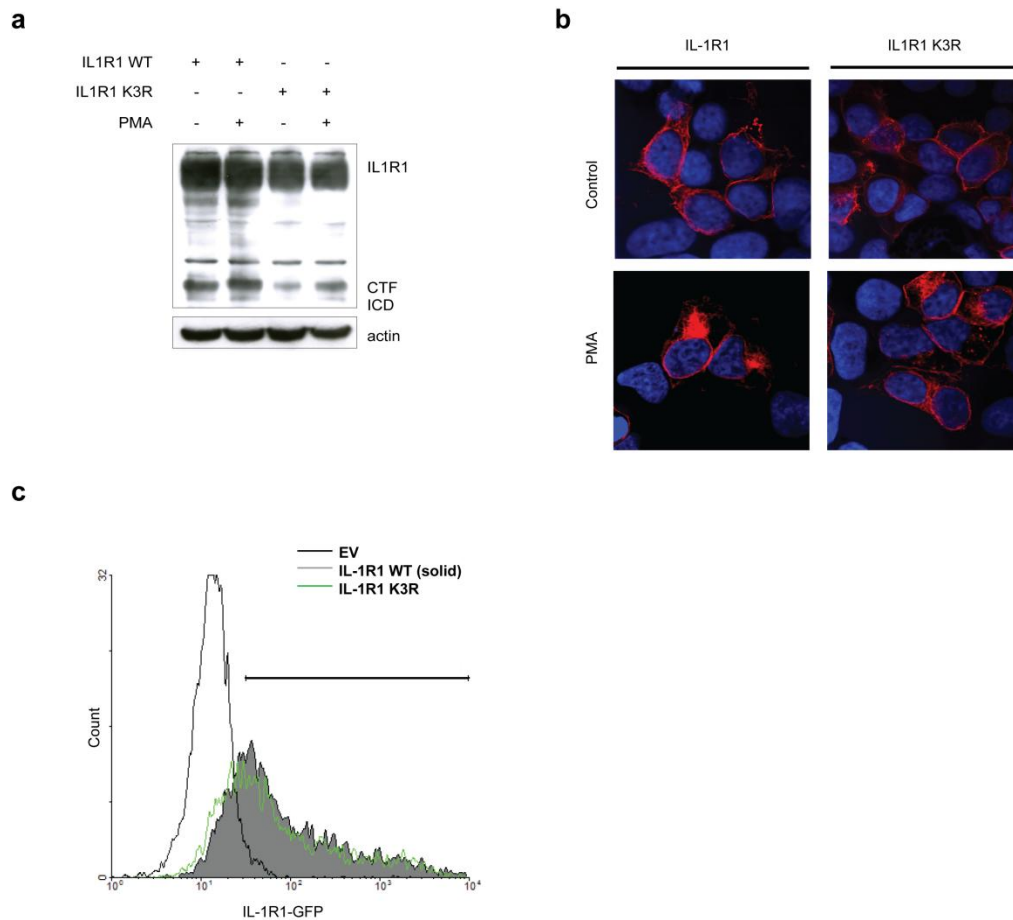


Figure 3.12 IL-1R1 K360/378/383R mutant is not defective in internalization

(a) Immunoblot analysis of IL-1R1 and IL-1R1 K360/378/383R mutant treated with PMA. **(b)** Immunofluorescence analysis HEK293T cells expressing IL-1R1 and IL-1R1 K360/378/383R mutant and stimulated with PMA to induce internalization and ectodomain shedding of IL-1R1. Cells were stained with anti-IL-1R1 antibody followed by Alexa Fluor 594. Samples were analysed using Olympus fluoview confocal microscope and fluoview FV1000 software. Red: IL-1R1; Blue: DAPI. **(c)** Cells expressing IL-1R1 and IL-1R1 K360/378/383R mutant were detached under nonpermeabilized condition and incubated with primary anti-IL-1R1 antibody for 45 minutes and secondary green fluorescent antibody for 30 minutes. After washings, levels of membrane IL-1R1 were measured by flow cytometry and are shown as histogram graphs. The data is representative of two independent experiments.

3.2.9 IL-1R1 ICD translocates to the nucleus

Certain γ -secretase cleavage products have been shown to produce biologically active receptor-derived ICDs, such as those observed for the Notch and p75NTR, which migrate to the nucleus and regulate gene expression (Fortini, 2002; Ilagan, 2004; Koo, 2004; Kopan, 2003). Thus, as the next step we studied the possible nuclear translocation of IL-1R1 ICD.

3.2.9.1 Immunofluorescence analysis to study nuclear translocation of IL-1R1 ICD

HEK293T cells were transfected with IL-1R1 and forty hours post-transfection cells were treated with IL-1 β (30ng/ml; 2h, 5h) and processed for immunofluorescence using C-terminus and N-terminus specific anti-IL-1R1 antibody, followed by Alexa Fluor 488. IL-1 β induced the formation of IL-1R1 CTF and IL-1R1 ICD with time (**Figure 3.13 a**). In contrast, no nuclear staining for IL-1R1 was seen in untreated cells or cells treated with IL-1 β and stained with C-terminus specific anti-IL-1R1 antibody (**Figure 3.13 b**). The ligand bound IL-1R1 is known to translocate to the nucleus (Curtis et al., 1990). Hence, cells were also stained with N-terminus specific anti-IL-1R1 antibody. The cells did not show nuclear staining for IL-1R1 (**Figure 3.113 c**). This data demonstrates the protocol or the cell system being used is not suitable. Thus, nuclear fractionation was carried out to check for the translocation of IL-1R1 ICD to the nucleus using PS wild-type mouse embryonic fibroblasts (PS WT MEFs) cells.

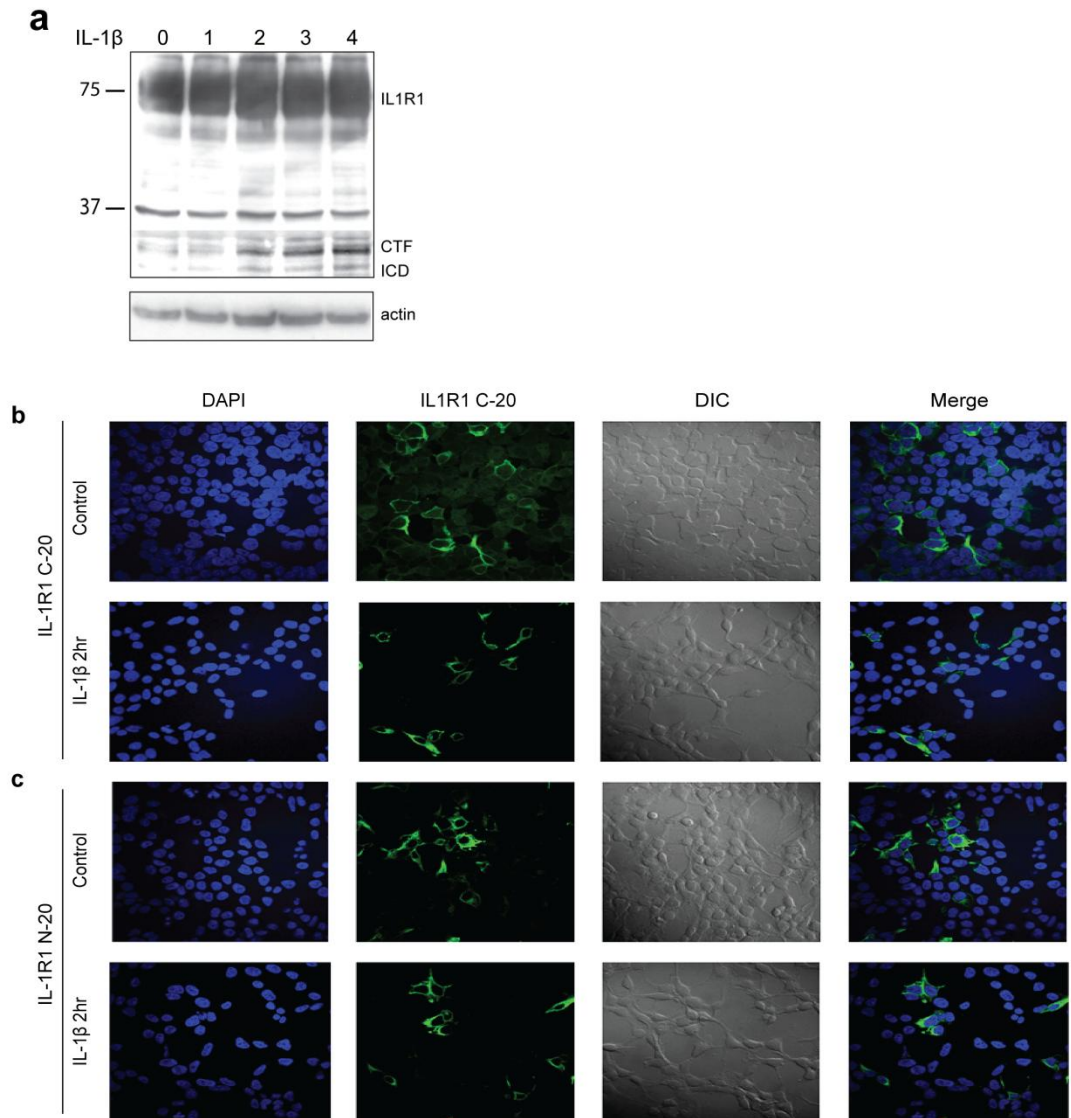


Figure 3.13 Immunofluorescence analysis to study nuclear translocation of IL-1R1 ICD

(a) HEK293T cells transfected with IL-1R1 and forty hours post-transfection stimulated with IL-1 β (30ng/ml) for indicated times. Immunoblot analysis revealed ligand induced ectodomain shedding and formation of IL-1R1 CTF and ICD. **(b)** Simultaneously cells were stained with N-terminal and C-terminal specific anti-IL-1R1 antibodies, to determine nucleic staining for the IL1R1. Samples were imaged using Olympus fluoview confocal microscope and fluoview FV1000 software. Green: IL-1R1; Blue: DAPI. The data is representative of two independent experiments.

3.2.9.2 PS1 is required for the γ -secretase-mediated formation of IL-1R1 ICD and its translocation to the nucleus

PS proteins are required for γ -secretase activity. Thus, wild-type PS (PSWT), PS1 knock-out (PS1KO), PS2 knock-out (PS2KO) and PS double-knock-out (PSDKO) MEFs were used to check for the translocation of IL-1R1 ICD to nucleus. PSWT MEFs over-expressing IL-1R1 were pre-treated with DAPT (10 μ M/ml; 8h) and stimulated with PMA (200ng/ml; 2h) subsequently the cells were processed for western blot analysis and nucleic fractionation. γ -secretase-mediated cleavage of IL-1R1 and subsequent IL-1R1 ICD formation was observed in PSWT and PS2KO MEFs (**Figure 3.14 a & 3.14c**). In contrast PS1KO MEFs were defective in γ -secretase-mediated cleavage of IL-1R1 (**Figure 3.14 b**). This data demonstrates that PS1 is required for γ -secretase-mediated cleavage of IL-1R1 and formation of IL-1R1 ICD formation. Simultaneously, these cells were processed for nucleic fractionation and analysed using western blots. Following PMA (200ng/ml; 2h) increased localization of IL-1R1 ICD in the nucleic fraction was observed (**Figure 3.14d**). Additionally, in PSWT and PS2KO MEFs, but neither in PS1KO nor in PSDKO MEFs, IL-1R1 ICD translocated to the nucleus (**Figure 3.14 e lane 3 to 6**). The observed IL-1R1 ICD in the nucleic fraction was inhibited on pre-treatment with DAPT (10 μ M/ml; 8h) (**Figure 3.14f**). Collectively the data demonstrates that PS1 is required for the γ -secretase-mediated formation of the IL-1R1 ICD, and that the IL-1R1 ICD translocates to the nucleus.

Figure 3.14 IL-1R1 ICD translocates to the nucleus

(a/b/c) Immunoblot analysis of PSWT, PS1KO and PS2KO MEFs, over-expressing IL-1R1. Forty hours post-transfection, cells were pre-treated with DAPT (20 μ M/ml; 8h) and treated with PMA (200ng/ml; 2h). **(d)** Subcellular fractionation of IL-1R1 in MEF PS WT cells untreated or treated with PMA, showing increased co-localization of IL-1R1 ICD with the nucleic fractionation upon PMA treatment. **(e)** Nucleic and cytosolic fraction of IL-1R1 transfected PSWT, PSDKO, PS1KO and PS2KO MEFs. **(f)** Nucleic fraction of IL-1R1 in MEF PSWT, PSDKO, PS1KO and PS2KO cells treated with PMA or PMA and DAPT. Anti-PARP (nucleic marker) and anti-tubulin (cytosolic marker) antibodies were used to check for the purity of the fraction. C-terminus specific anti-IL-1R1 antibody was used to assess the cleavage profile of the receptor. The data is representative of three independent experiments.

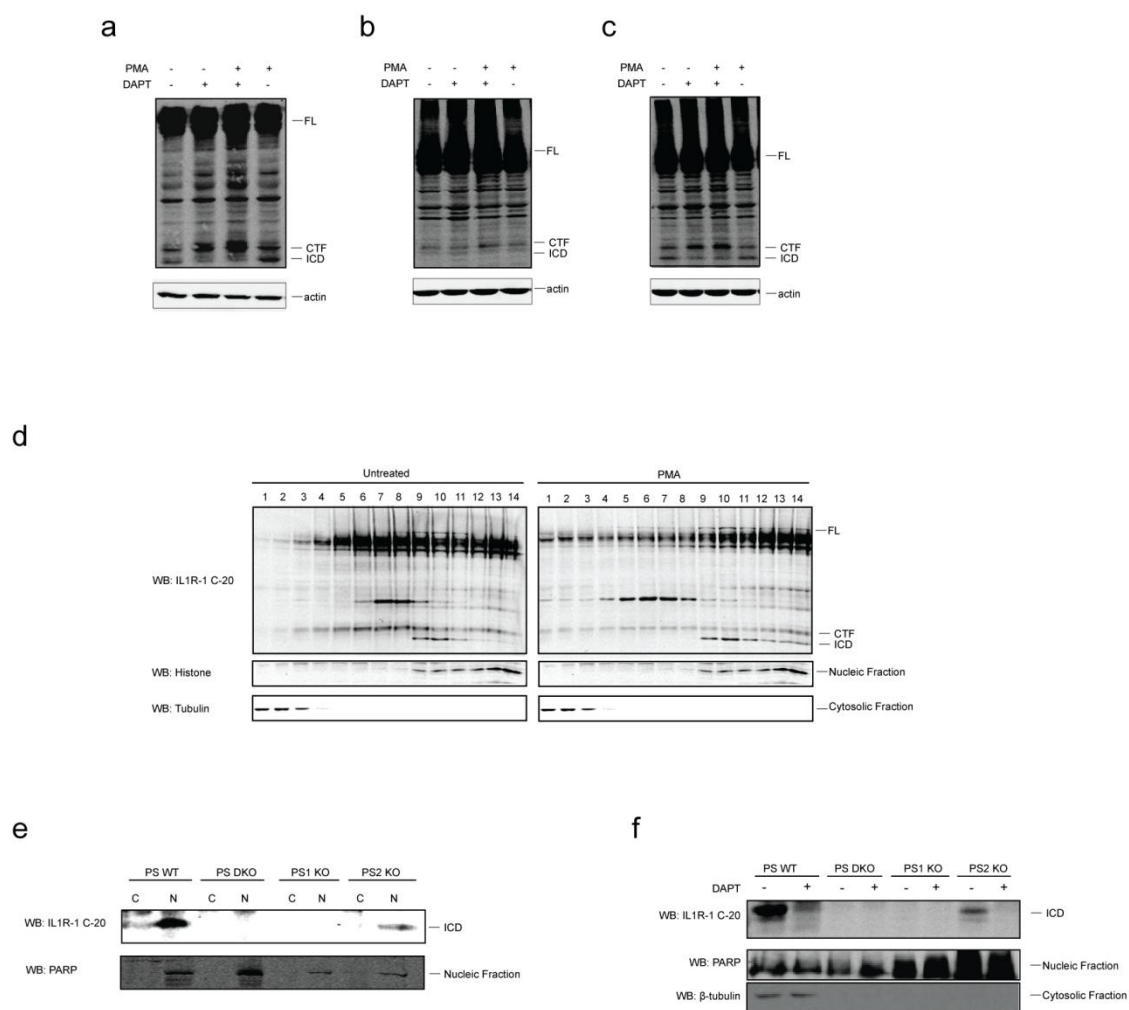


Figure 3.14 IL-1R1 ICD translocates to the nucleus

3.2.10 IL-1R1 ICD has four nuclear localization signal sequences

A nuclear localization signal (NLS) is a short stretch of amino acid which mediates the transport of proteins into the nucleus. Positively charged residues are abundant in NLS sequences and mutating these residues has been reported to disrupt the nuclear import of proteins. The Nuclear Localization Predictor program available online was used along with alignment programs to predict the presence of NLS sequences within the ICD of IL-1R1. **Figure 3.15** shows the alignment of IL-1R1 ICD across species and highlights the presence of several highly conserved putative NLS sequences. To investigate the importance of these residues in translocation of IL-1R1 ICD into the nucleus, the highly positively charged lysine residues within these NLS sequences were mutated to arginine. These lysine residues were also specifically targeted based on the previous mutational studies done on IL-1R1, listed in the **Table 1.6**. The residues K445/446, K527, K527/532, and K548 were mutated and further characterized to study their effect on nuclear import of IL-1R1 ICD.

IL-1R1 across species:

	667	696
HUMAN IL1R 1 (434)	EDIVEVINENVKKSRRLLIIILVRETSGFSW	
IL1R 1 Chimp (434)	EDIVEVINENVKKSRRLLIIILVRETSGFSW	
IL1R I Dog (644)	EDIVEVANENIRKSRRLIIILVPETSGLC	
IL1R I Rat (451)	EDTIEVTNENVKR SRRLIIILVRDMGSFSC	
murine IL1 (437)	EDTIEVTNENVKKSRRLLIIILVRDMGGFSW	
Consensus (667)	EDIVEV NENVKKSRRLLIIILVRETSGFSW	
	R 431	
	EDIVEV NENVKKSRRLLIIILVRETSGFSW	
	NLS1	

HUMAN IL1R 1 (523)	PQSAKTRFWKNVRYHMPVQRRSPSSKHQLLSPA
IL1R 1 Chimp (523)	PQSAKTRFWKNVRYHMPVQRRSPSSKHQLLSPA
IL1R I Dog (733)	LQSAKTRFWKNVRYHMPVQRQPSKHSLSLA
IL1R I Rat (540)	PQSAKTRFWKNLRYQMPAQRRSPLSKHLLTLD
murine 1L1 (526)	PQSAKTRFWKNLRYQMPAQRRSPLSKHLLTLD
Consensus (756)	PQSAKTRFWKNVRYHMPVQRRSPSSKHQLLSLA
	K515 R518
	PQSAKTRFWKNVRYHMPVQRRSPSSKHQLLSLA
	NLS4 NLS3 NLS2

	Mature murine IL-1R1	Immature murine IL-1R1
NLS 1	429-433	448-452
NLS 2	507-519	526-538
NLS 3	523-527	542-546
NLS 4	529-534	548-553

Mature murine IL-1R1	Immature murine IL-1R1
R 431	R 448
K 515	K 532
K 518	K 535
Mature human IL-1R1	Immature human IL-1R1
R 431	R 448
K 513	F 530
W 514	W 531
K 515	K 532
R 518	R 535
Y 519	Y 536
P 522	P 539

Figure 3.15 Alignment of IL-1R1 ICD across a number of species

Schematic representation of the four IL-1R1 point mutants constructed in our attempt to validate the NLS sequence responsible for translocation of IL-1R1 ICD into the nucleus.

3.2.11 Mutating residues within the IL-1R1 NLS sequences does not alter the translocation of IL-1R1 ICD into the nucleus

HEK293T cells were transfected with either the wild-type IL-1R1, IL-1R1 K445/446R, IL-1R1 K527R, IL-1R1 K527/532R or IL-1R1 K548R mutants and forty hours post-transfection pre-treated with DAPT (20 μ M/ml; 8h) and subsequently with PMA (200ng/ml; 2h). All these mutants- IL-1R1 K445/446R, IL-1R1 K527R, IL-1R1 K527/532R responded to PMA and DAPT treatment similar to cells expressing wild-type IL-1R1, with clear detection of CTF and ICD fragments (**Figure 3.16**). These constructs were also examined for altered subcellular localization compared to the wild-type IL-1R1. HEK293T cells were plated on coverslips and transfected with the wild-type IL-1R1 and the four NLS sequence mutants and co-transfected with either Rab5 or Rab7 to stain for the early and late endosomes respectively. Cells were fixed and permeabilized and labelled with C-terminus specific anti-IL-1R1 antibody, followed by Alexa Fluor-594; and DAPI to stain the nucleus. The subcellular localization of IL-1R1 K445/446R and IL-1R1 K548R mutants remained unchanged, however, the IL-1R1 527R and IL-1R1 527/532R mutants showed increased co-localization with the late endosomes (**Figure 3.17**). These mutants were also analyzed for nuclear translocation of the ICD using fractionation. Immunoblot analysis of the nucleic fractions revealed that IL-1R1 K445/446R and K527/532R mutants did not show any defective nuclear localization of ICD, in contrast there was decreased ICD detected in the nuclear fraction in cells expressing K527R mutant. However, no IL-1R1 K548R ICD was seen in nuclear fraction (**Figure 3.18 lane 9 &10**).

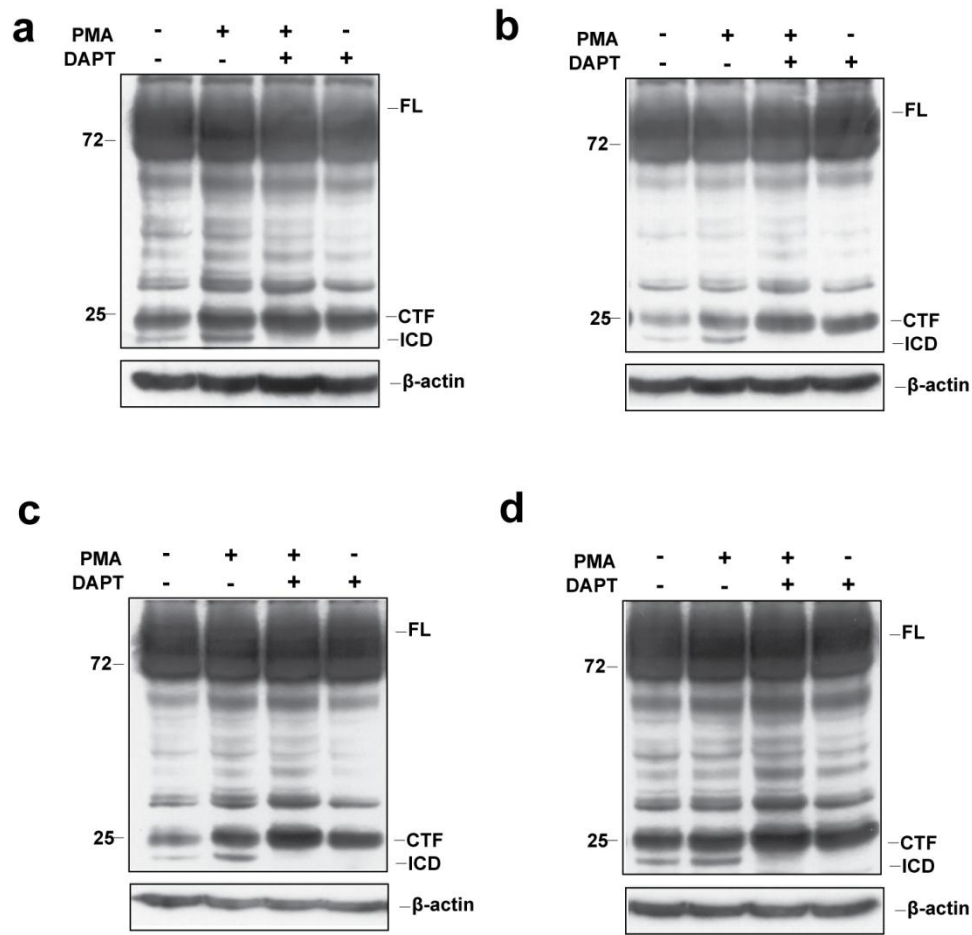


Figure 3.16 Response of IL1-R1 NLS sequence mutants to PMA and DAPT

HEK293T cells were transfected with the indicated constructs and forty hours post-transfection, pre-treated with DAPT, followed by treatment with PMA. Cell lysates were analysed using immunoblots. C-terminus specific anti-IL-1R1 antibody was used to assess the cleavage profile of the receptor. **(a)** IL-1R1 K445/446R **(b)** IL-1R1 K527R **(c)** IL-1R1 K527/532R **(d)** IL-1R1 K548R The data is representative of two independent experiments.

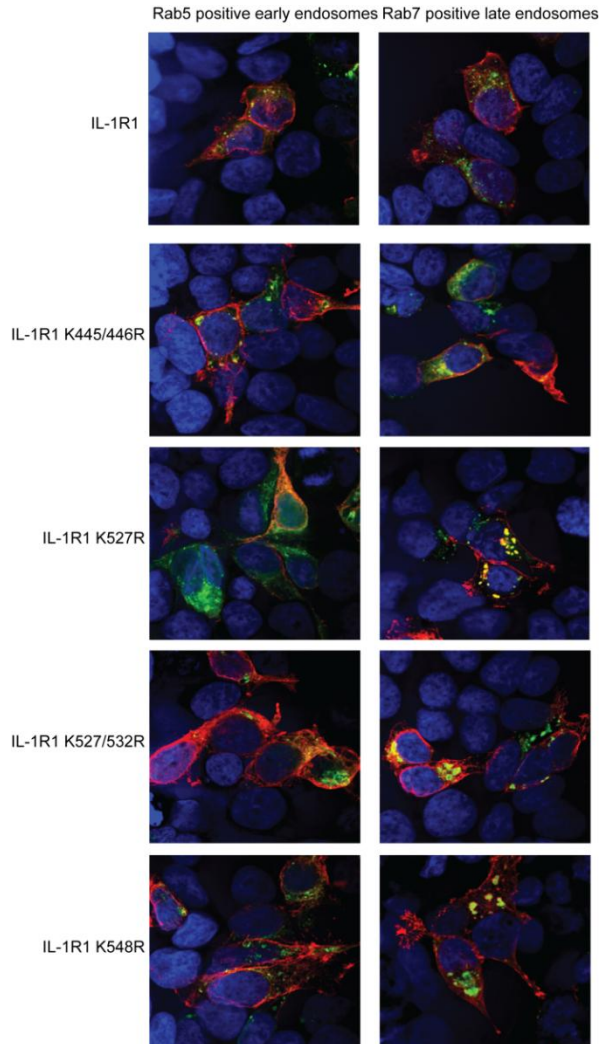


Figure 3.17 Subcellular localization and trafficking of IL-1R1 NLS sequence mutants

HEK 293T cells growing on coverslips were transfected with wild-type IL-1R1, IL-1R1 K445/446R, IL-1R1 K527R, IL-1R1 527/532R and IL-1R1 548R, and co-transfected with early endosome marker (Rab5) and late endosome marker (Rab7). Cells were then fixed, permeabilized and stained using C-terminus specific anti-IL-1R1 antibody. Increased co-localization of IL-1R1 K527R and IL-1R1 K527/532R was seen with the late endosomes, while other mutants showed no trafficking defect. (Red: IL-1R1; Green: Rab5 and Rab7 positive vesicles; Blue: DAPI-nucleus) The data is representative of two independent experiments.

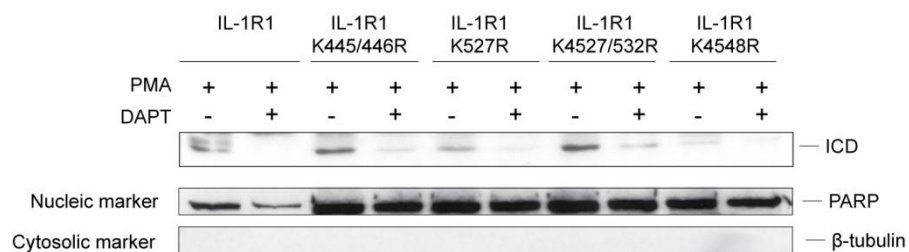


Figure 3.18 Nucleic translocation of wild-type IL-1R1 and IL-1R1 NLS sequence mutants

Nucleic fraction of wild-type IL-1R1 and IL-1R1 NLS sequence mutants transfected HEK293T cells treated with PMA alone or in combination with DAPT. Anti-PARP (nucleic marker) and anti-tubulin (cytosolic marker) antibodies were used to check for the purity of the fraction. C-terminus specific anti-IL-1R1 antibody was used to assess the cleavage profile of the receptor. The data is representative of two independent experiments.

3.2.12 TLR4 is a substrate for γ -secretase mediated regulated intramembrane proteolysis

Identification of members of the IL-1/TLR superfamily, for instance IL-1R1, IL-1R2, and IL-6R as substrates for γ -secretase-mediated regulated intramembrane proteolysis (Chalaris et al., 2010; Elzinga et al., 2009; Kuhn et al., 2007), prompted us to investigate whether or not other members of the family, particularly TLR4 was a potential γ -secretase substrate.

HEK293T cells were transfected with the C-terminus HA-epitope-tagged TLR4 construct, and forty-eight hours post-transfection cells were pre-treated with DAPT (20 μ M/ml; 8h) or DMSO as a vehicle control in serum-free media and subsequently with PMA (200ng/ml; 2h). Cells were harvested and lysed using RIPA lysis buffer. Immunoblot analysis showed that PMA-induced formation of two detectable fragments that corresponded in size to a putative TLR CTF and ICD fragment (**Figure 3.19 lane 1 & 4**). Importantly formation of the putative TLR4 ICD fragment was significantly decreased in the presence of DAPT (**Figure 3.19a lane 2 & 3**). Densitometry analysis of the TLR4 ICD showed significant decrease in the TLR4 ICD formation in presence of DAPT (**Figure 3.19**). Thus, verifying that TLR4 is a substrate for γ -secretase-mediated cleavage.

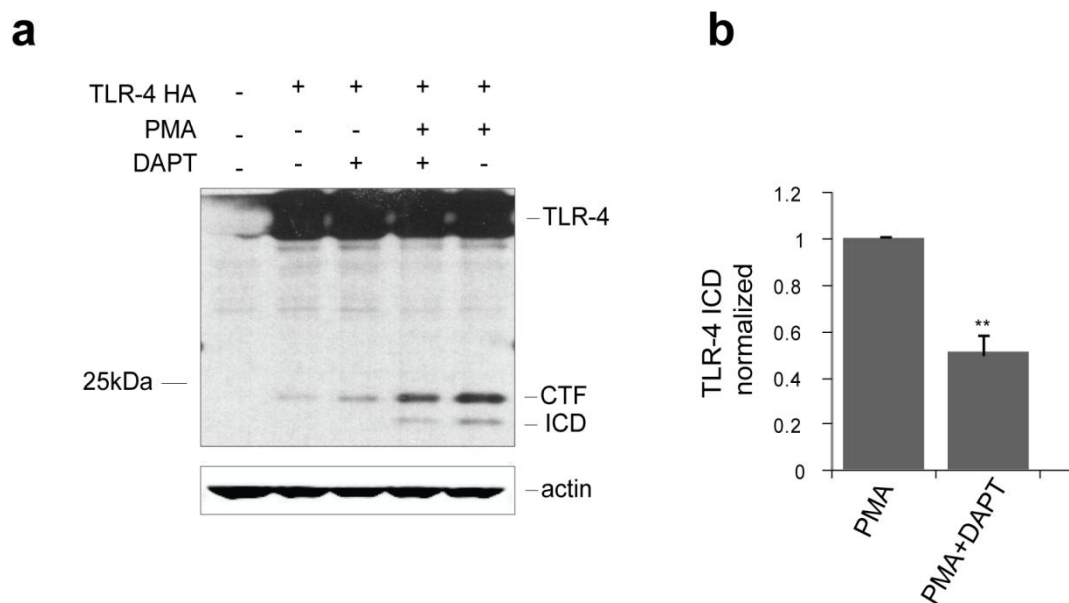


Figure 3.19 TLR4 is a γ -secretase substrate

(a) HEK293T cells transiently expressing TLR4 HA, forty-eight hours post-transfection were pre-treated with DAPT (20 μ M/ml; 8h) followed by treatment with PMA (200ng/ml; 2h). Immunoblots were probed with anti-HA tag antibody. **(b)** Densitometry analysis of TLR4 ICD normalised to total β -actin for all experimental conditions. The amount of TLR4 ICD is expressed as TLR4 ICD immunoreactivity \pm standard error of the mean, representative of three independent experiments.

3.2.13 TLR4Fas-TM construct does not undergo γ -secretase-mediated proteolysis

The p75NTR is a well characterized γ -secretase substrate, while the Fas receptor is not a substrate for γ -secretase-mediated cleavage. Kong and co-workers employed a novel experimental approach to identify which domains of p75NTR were required for γ -secretase cleavage. In this study they generated a p75NTR chimeric receptor in which they exchanged the transmembrane domain of p75NTR with transmembrane domain of the Fas receptor, and in doing so created a non-cleavable p75NTR (Kong et al., 1999). We employed the same approach and replaced the transmembrane domain of TLR4 with the transmembrane domain of the Fas receptor, to generate a chimeric TLR4Fas-TM construct. To determine if TLR4Fas-TM chimeric receptor was indeed resistant to γ -secretase-mediated cleavage, HEK293T cells were transiently transfected with TLR4Fas-TM chimeric receptor and stimulated with PMA (200ng/ml; 2h) and analysed using western blot to check for the formation of the γ -secretase-cleavage dependent TLR4 ICD. Immunoblot analysis revealed that upon PMA treatment wild-type TLR4 undergoes ectodomain shedding as seen by increased generation of TLR4 CTF and ICD (**Figure 3.20 lane 1 & 2**), in contrast the TLR4Fas-TM construct did undergo PMA-mediated ectodomain shedding as evident from increased detection of TLR4 CTF, but it was defective in γ -secretase-mediated cleavage and subsequent formation of the ICD (**Figure 3.20 lane 3 & 4**).

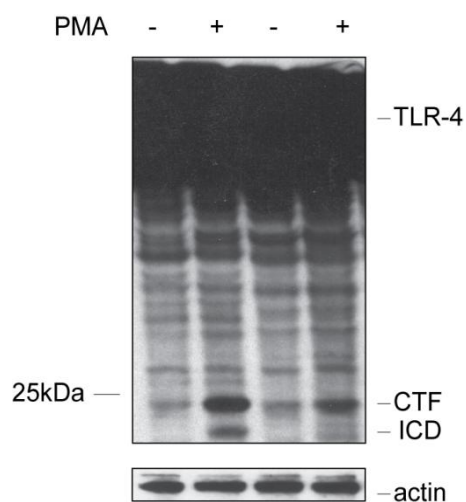


Figure 3.20 TLR4Fas-TM construct does not undergo γ -secretase-mediated proteolysis

HEK293T cell transiently expressing wild-type TLR4 and TLR4 Fas-TM receptor, forty-eight hours post-transfection were treated with PMA (200ng/ml; 2h), lysed and probed with anti-HA tag antibody to assess the cleavage profile of TLR4. The data is representative of two independent experiments.

3.3 Discussion

3.3.1 IL-1R1 endocytosis is required for ectodomain shedding of IL-1R1

IL-1R1 plays an important role in innate immunity and inflammatory responses. On binding to IL-1 β , IL-1R1 forms a complex with the IL-1RAcP and initiates two different signalling pathways: cell surface-TAK1-dependent and cytosolic-TAK1-independent (Subramaniam et al., 2004). In an independent study we found that IL-1R1 undergoes ADAM17-mediated ectodomain shedding (unpublished). Gaining more knowledge about the spatiotemporal localization of ADAM17-mediated cleavage of IL-1R1 will help us to understand the importance and subsequent signalling pathways that it affects. In this context, using a pharmacological inhibitor: dynasore, and a dominant negative mutant of dynamin, Dyn-DN, we demonstrated that IL-1R1 undergoes ectodomain shedding on the early endosomes (**Figure 3.3, 3.4 and 3.5**). This was further confirmed using the dominant active Rab5 DA and dominant active Rab5 DN mutants, whereby we showed that Rab5 DN prevented formation of early endosomes and inhibited the formation of IL-1R1 CTF. In contrast, Rab5 DA enhanced the fusion of endocytic vesicles and formation of the early endosomes, thus leading to increased IL-1R1 CTF and subsequently formation of the IL-1R1 ICD (**Figure 3.6**).

Inhibiting receptor internalization using a pharmacological inhibitor or dominant negative mutants raises the possibility of an indirect effect of inhibiting the membrane trafficking in the cell system. Thus, we also mapped a highly conserved internalization motif in IL-1R1, mutagenesis of which inhibited constitutive and PMA-induced ectodomain shedding of IL-1R1 (**Figure 3.7**). The IL-1R1 W262A mutant did not express to the same levels as the wild-type IL-1R1. Despite of low expression levels it was significantly clear that IL-1R1 W262A mutant was defective in ectodomain shedding and CTF formation (**Figure 3.8**). Our demonstration that unlike many other receptors such as TNFR1, TNFR2 and p75NTR, ectodomain shedding of IL-1R1 occurs on the

endosome highlights that it may play an important role in the IL-1R1-mediated endosomal signalling pathways. This is of particular interest since most released ectodomains in addition to regulating the normal turnover of the receptor also retain their biological activities and it is feasible that before being secreted into the extracellular milieu sIL-1R1 participates in mediating signalling events. Additionally, on the basis of a study carried out by Heguy and co-workers showing that ligand bound C-terminal domain truncated IL-1R1 migrates to the nucleus (Heguy et al., 1991), it is possible for sIL-1R1 to translocate to the nucleus and participate in gene regulation.

3.3.2 TRAF6 E3 ligase enhances constitutive and PMA-induced ectodomain shedding

In the current study we have clearly shown that IL-1R1 ectodomain shedding occurs on the early endosomes and that IL-1R1 ICD undergoes proteasomal degradation (**Figure 3.9**). In a recent study carried out by Hansen and co-workers, inhibition of dynamin-dependent endocytosis was shown to deregulate IL-1 β induced signalling. They suggested a novel pathway requiring IL-1R1 endocytosis for IL-1 β -induced NF- κ B-dependent gene expression downstream of nuclear translocation and DNA binding of NF- κ B (Hansen et al., 2013). Previously our group had proposed a molecular mechanism whereby TRAF6 promotes ubiquitination and regulated intramembrane proteolysis of IL-1R1 through its ubiquitin ligase activity. In brief, TRAF6 was shown to preferentially induce ubiquitination of IL-1R1. Furthermore, TRAF6 ubiquitin ligase activity and ubiquitination of IL-1R1 were positively correlated with IL-1R1 ectodomain shedding and γ -secretase-mediated cleavage (Twomey et al., 2009).

We have subsequently shown that the TRAF6 ubiquitination sites are restricted to the juxtamembrane residues 360, 378, 383 and IL-1R1 triple lysine mutant (IL-1R1 K360/378/383R) is defective in TRAF6-mediated polyubiquitination of IL-1R1 CTF (**Figure 3.11**) and is further defective in constitutive and PMA-induced ectodomain shedding. However, mutating the three lysine residues did not affect internalization of

the receptor. This is suggestive of a role for TRAF6-mediated ubiquitination of IL-1R1 in mediating ectodomain shedding and γ -secretase-mediated cleavage.

3.3.3 IL-1R1 ICD translocates to the nucleus

As previously discussed γ -secretase-mediated cleavage of several substrates plays an important role in regulating their signalling events. It either functions to clear the CTF from the membrane, thereby terminate the signalling events. Also, for substrates like, APP, p75NTR and Notch the ICD generated translocates to the nucleus and drives transcription of various genes. Previously, IL-1 β /IL-1R1 complex has been shown to translocate to the nucleus. However, deletion of the C-terminus domain of IL-1R1 did not affect its nuclear translocation. The role played by the IL-1 β /IL-1R1 complex within the nucleus has been studied but remains unclear. Thus, the possibility that IL-1R1 ICD translocates to the nucleus was explored.

The limitation of this study was lack of IL-1R1 ICD specific antibody. Attempts to map the γ -secretase cleavage site within the transmembrane domain of IL-1R1 failed. Two different approaches were used. Firstly point mutants of the highly conserved site within the transmembrane domain were made, which failed to create a non-cleavable IL-1R1 receptor. Høgl and co-workers used mass spectroscopy in order to determine the cleavage sites of APLP by ADAM10, BACE1 and γ -secretase, (Høgl et al., 2011). Using the same approach, an attempt was made to map the γ -secretase cleavage site for IL-1R1. However, due to low expression of the IL-1R1 ICD, it was not successful. Having failed to map the cleavage site, GFP-tagged IL-1R1 construct was made. However, the GFP-tag was cleaved from the IL-1R1 and degraded. Therefore, no immunofluorescence studies were carried out in HEK293T cells over-expressing the wild-type IL-1R1 or the IL-1R1 NLS sequence mutants.

Using PS WT MEFs, subcellular fractionation studies revealed that IL-1R1 ICD translocates to the nucleus (**Figure 3.14**). Sequence alignments, and using NLS sequence predictor tools, four NLS sequences were found in the ICD of IL-1R1 (**Figure 3.15**). These NLS sequences contained residues which had been previously studied and shown to either drastically affect trafficking or expression of IL-1R1. Hence, these residues were not targeted for mutagenesis in this study. For the other residues, NLS predictor program was used and residues-445, 446, 527, 532 and 548 were chosen and mutated to arginine. The residue 527, when mutated affected the trafficking of IL-1R1 as seen by increased co-localization with the early endosome (**Figure 3.17**). The mutation of the lysine residue 445/446 to arginine did not affect translocation of IL-1R1 ICD, in contrast reduced levels of ICD were observed in nucleic fraction of cells over-expressing IL-1R1 K527/532R mutant (**Figure 3.18**). However, this could be an indirect effect of defective trafficking of this mutant. On the other hand in cells expressing IL-1R1 K548R mutant no ICD was observed in nucleic fraction. We suggest that a more quantitative approach is required to validate this result and determine the role played by IL-1R1 ICD in the nucleus. Parkhurst and co-workers studied the nuclear translocation of p75NTR ICD using nuclear translocation assay and biochemical fractionation (Parkhurst et al., 2010). This group fused C-terminus domain of p75NR ICD with the LexA DNA-binding domain-Gal4 transactivation domain (LG), which was transfected in the cells. The LG sequence is capable of activating a GFP reporter gene controlled by eight LexA operator sequences. Nuclear translocation is required to transactivate the LexA operator sites of the reporter gene. Using a similar approach nuclear translocation of IL-1R1 ICD in the NLS sequence mutant K548R could be further verified.

3.3.4 TLR4 is a substrate for γ -secretase-mediated endosomal regulated intramembrane proteolysis

In this study, TLR4 was shown to undergo constitutive and PMA-induced ectodomain shedding followed by γ -secretase-mediated regulated intramembrane proteolysis. Identification of TLR4 as a γ -secretase substrate reflected a novel role of PS in regulating LPS-induced immune responsiveness. This was investigated in detail by our group and surprisingly γ -secretase-independent role of PS2 was seen in LPS-induced cytokine and interferon production both *in-vitro* and *in-vivo* (**refer to manuscript**). Similar to IL-1R1, members of the TLR superfamily regulate the ligand induced signalling events via compartmentalization. TLR4-mediated signalling events have been shown to be spatially segregated, where TLR4 recruits TRIF and MyD88 on the cell surface, followed by endosomal recruitment of TRAM and TRIF (Guillot et al., 2004; Kagan et al., 2008). The molecular mechanisms underlying this spatial segregation remain elusive. Experiments performed to determine the subcellular localization for γ -secretase-mediated cleavage of TLR4 could provide insight into the role played by this cleavage in spatially segregated TLR4-mediated signalling events. Preliminary experiments were carried out to screen C-terminus specific TLR4 antibody suitable for immunofluorescence. C-terminus specific TLR-4 antibody (sc-8694-R) revealed that dominant negative Rab5 construct defective in fusion of vesicles and formation of early endosomes prevented trafficking of TLR4 (**Figure 3.21**). This could help investigate the subcellular localization for TLR4 cleavage and further study the nuclear localization of TLR4 ICD similar to IL-1R1.

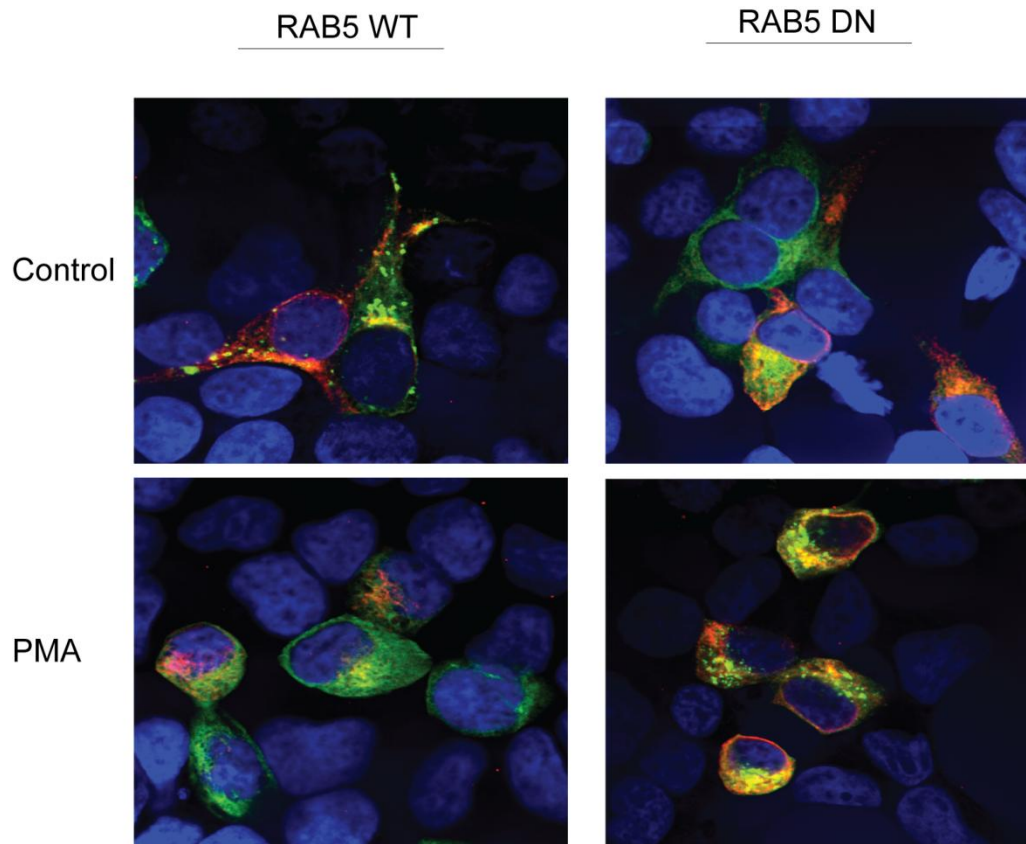


Figure 3.21 γ -secretase-mediated cleavage of TLR4 occurs after exit from the early endosome

Immunofluorescence analysis of corresponding cells grown on coverslips, expressing TLR4 and co-transfected with GFP-tagged Rab5 WT or Rab5 DN constructs. Cells were stimulated with PMA (200ng/ml; 2h), fixed, permeabilized and stained with C-terminus specific anti-TLR4 antibody. Samples were imaged using Olympus fluoview confocal microscope and analysis using Fluoview 5000. Red: TLR4; Green: Rab5 positive vesicles; Blue: DAPI.

CHAPTER 4

TNFR1 is a substrate for γ -secretase-mediated regulated intramembrane proteolysis

4.1 Introduction

The γ -secretase enzyme complex was initially characterized by its involvement in A β peptide generation and subsequently found to mediate cleavage of the Notch receptor, an essential proteolytic event in the propagation of Notch signalling (Strooper et al., 1999; Fortini, 2002; Hub et al., 2001; Kopan, 2003). To date, over 90 distinct γ -secretase substrates have been identified indicating that γ -secretase has a generic role in the processing of membrane proteins (Haapasalo, 2012). From culminating research, it is now evident that the γ -secretase protease functions to target many of its substrates for degradation; for example, the cleavage of CD43 by γ -secretase terminates CD43-initiated signalling events facilitating its clearance from the plasma membrane (Andersson et al., 2005). For other substrates, cleavage by γ -secretase leads to the generation of biologically active receptor-derived ICDs, such as those observed for the Notch, and p75NTR, which migrate to the nucleus and regulate gene expression (Fortini, 2002; Ilagan, 2004; Koo, 2004; Kopan, 2003).

The PS proteins are the catalytic component of this multiprotein γ -secretase complex (Hass et al., 2009; Wilson et al., 2002). Genetic inactivation of PS1 alone or both PS1 and PS2 results in a marked reduction, or complete loss of this γ -secretase activity (Strooper et al., 1998; Herreman et al., 2000; Wolfe et al., 1999). Various groups have demonstrated that expression of FAD-linked PS variants are either directly cytotoxic or enhance susceptibility to apoptosis (Deng et al., 1996; Parent, 2004; Wolozin et al., 1998). Consistent with this, PS null mice display many abnormalities, including gross deformities of the axial skeleton and massive neuronal loss (Donoviel et al., 1999). These have been credited to increased susceptibility to apoptosis owing to which these animals die shortly after birth (Hartmann et al., 1999). This phenotype has always been attributed to defective Notch signalling following inhibition of γ -secretase activity (Curry et al., 2005; Rosati et al., 2013). However, the involvement of specific cell death receptors as potential γ -secretase substrates has not been studied.

Work in our group had previously demonstrated a role for PS1 in TNF-mediated intracellular signalling (unpublished data). Among the members of the TNF superfamily, TNFR1 is most widely studied due to its ability to sequentially activate an NF- κ B-mediated cell survival pathway and also signal for cell death by activating caspases (Schneider-Brachert et al., 2004). The predominance of TNFR1 signalling is also reflected by its ubiquitous expression in most cell types. Three ligands are selectively responsible for the activation of TNFR1; the membrane bound TNF α , soluble TNF α , and the soluble lymphotoxin- α (LT- α)/TNF- β .

In 2006, Fluhner *et al.* reported that the TNFR1 ligand, TNF α , undergoes regulated intramembrane proteolysis via the action of the iCLIP family member, signal peptide peptidase like protein, SPPL2b (Fluhner et al., 2006). SPPL2b releases the ICD of TNF α into the cytosol and the carboxy-terminal region is secreted into the extracellular milieu similar to γ -secretase processing. Furthermore, the TNF receptor superfamily member, p75NTR is a very well characterized substrate for γ -secretase (Frade et al., 2010; Skeldal et al., 2011), enabling the generation of the p75NTR ICD which translocates to the nucleus and is reported to regulate gene transcription (Kanning et al., 2003).

TNFR1 fits the profile of the majority of known γ -secretase substrates, being a type-1 transmembrane receptor, with an extracellular N-terminal and an intracellular C-terminal domain, it is subjected to TACE-mediated ectodomain shedding, a characteristic feature of γ -secretase substrates (Black et al., 1997; Peschon, 1998). These characteristics suggest that it is feasible for the membrane-bound TNFR1 CTF formed as a result of ectodomain shedding to be subsequently processed by γ -secretase.

The aim of this chapter is twofold-

1. Determine whether or not TNFR1 is a γ -secretase substrate.
2. Characterize the subcellular location of TNFR1 proteolysis and determine the biological relevance of TNFR1 regulated intramembrane proteolysis.

4.2 Results

4.2.1 Expression of exogenous TNFR1

Activation of TNFR1 by TNF α initiates several distinct intracellular signalling pathways that can either lead to activation of the MAPKs and the transcription factor NF- κ B, or the induction of apoptosis (Verstrepen et al., 2008). Exogenous expression of TNFR1 is known to induce apoptotic cell death, thereby making *in vitro* cell culture studies difficult. In order to prevent cell death on over-expression of TNFR1, we used a TNFR1 mutant, TNFR1 L380A, which has a point mutation in the DD and like the DD inactivating *lpr*^{cg} mutation in Fas, prevents the induction of apoptosis following ectopic expression in cells (Boone et al., 2000). In this study the TNFR1 L380A mutant is referred to as TNFR1 to distinguish it from other TNFR1 mutants employed in the study unless otherwise specified.

To examine the expression of TNFR1 and verify that it undergoes ectodomain shedding, HEK293T cells were transiently transfected with TNFR1 and thirty-four hours post-transfection, cells were treated with PMA (200ng/ml; 2h) in serum-free media to promote TACE-mediated ectodomain shedding. The expression and cleavage profile of TNFR1 was analysed by immunoblotting, using anti-TNFR1 C25 antibody raised against the C-terminal epitope 431-455. TNFR1 expressed to comparable levels in all experimental conditions (**Figure 4.1a**). The generation of a constitutive and PMA-induced TNFR1 CTF was readily detected in cell lysates. In cultures stimulated with PMA, increased formation of an additional band of ~32-kDa was clearly detected. This band most likely corresponds to the TNFR1 ICD formed by γ -secretase-mediated cleavage of TNFR1 (**Figure 4.1a**).

To confirm PMA-induced ectodomain shedding of TNFR1, conditioned media from the cell cultures was collected and analysed for sTNFR1 using a sTNFR1 specific ELISA. PMA

stimulation induced significant ectodomain shedding and generation of sTNFR1 (**Figure 4.1b**). Similarly, flow cytometry analysis of cell surface TNFR1 revealed a decrease in TNFR1 cell surface levels following PMA treatment (**Figure 4.1c/d**). Thus, the expression of the exogenous TNFR1 and its responsiveness towards PMA was confirmed in the cell system being used.

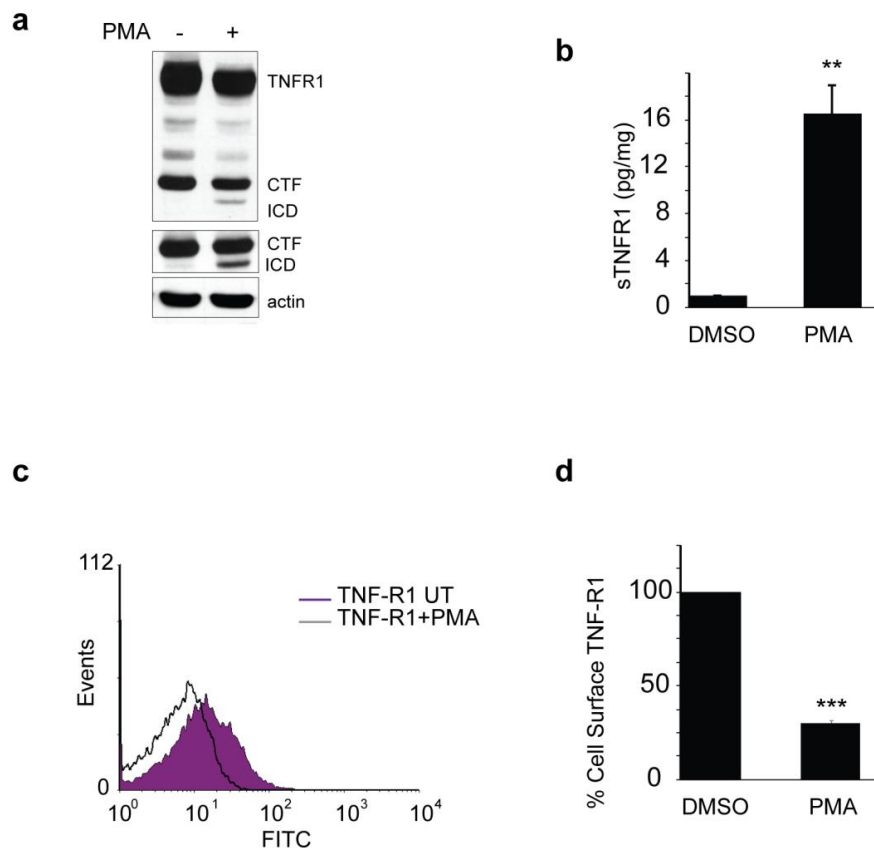


Figure 4.1 Expression and localization of TNFR1

(a) Whole cell lysates were immunoblotted with a C-terminus specific anti-TNFR1 antibody to confirm expression of exogenous TNFR1 and to allow assessment of its cleavage pattern following treatment with PMA. **(b)** Analysis of conditioned media for sTNFR1 from cell cultures treated with PMA, using sTNFR1 specific ELISA (Invitrogen). Data are expressed as picogram of sTNFR1 per milligram of total protein \pm standard error of the mean, representative of three independent experiments versus TNFR1 control. Asterisks indicate significance to the control- DMSO. **(c)** Cell surface levels of TNFR1 measured by flow cytometry, performed with AccuriC6, and analysed using Accuri FlowJo software. **(d)** Relative cell surface levels of TNFR1 in untreated and PMA treated cells, and statistical analysis was performed using unpaired Student's *t*-test. *, $p < .05$; **, $p < .01$; ***, $p < 0.001$. Asterisks indicate significance to the control-DMSO. All these experiments were repeated three times.

4.2.2 Identification of TNFR1 as a γ -secretase substrate

Two different approaches were used to identify TNFR1 as a potential γ -secretase substrate. First, DAPT, a pharmacological inhibitor of γ -secretase, was used. Second, we used a genetic approach employing wild type (WT) and PS double-knock-out (PS DKO) murine embryonic fibroblasts (MEFs), which possess no γ -secretase catalytic activity due to the absence of both PS1 and PS2 (Herreman et al., 2000).

4.2.2.1 Pharmacological inhibition of γ -secretase activity

HEK293T cells were transiently transfected with TNFR1 construct and twenty-eight hours post-transfection cells were pre-treated with DAPT (10 μ m/ml; 8h) or DMSO as a vehicle control in serum-free media. Cells were subsequently stimulated with PMA (200ng/ml; 2h) or recombinant human TNF α (hTNF α) (30ng/ml; 2h), alone or in combination with DAPT (**Figure 4.2**). The γ -secretase substrate IL-1R1 was included as a positive control in this experiment (Elzinga et al., 2009).

The cleavage profiles of TNFR1 and IL-1R1 were examined by immunoblotting with their respective C-terminus antibody (TNFR1 C25 and IL-1R1 C20). Immunoblot analysis revealed that TNFR1 underwent constitutive and PMA-induced proteolytic cleavage as evident from the detection of proteolytic fragments, which based on molecular weights were predicted to correspond to TNFR1 CTF, generated following TNFR1 ectodomain shedding and ~32-kDa TNFR1 ICD (**Figure 4.2a lane 1 & 2**). Pre-treatment of cell cultures with the γ -secretase inhibitor, DAPT, completely suppressed the formation of this ~32-kDa TNFR1 ICD (**Figure 4.2a lane 4**), suggesting that it was indeed the product of γ -secretase-mediated cleavage of TNFR1 CTF. In cells treated with both PMA and DAPT no TNFR1 ICD fragment was detected (**Figure 4.2a lane 3**), however a parallel increased accumulation of the TNFR1 CTF was observed. This responsiveness to PMA and DAPT is consistent with the cleavage profile of other γ -secretase

substrates (Haapasalo et al., 2012), indicating a precursor product relationship, whereby inhibition γ -secretase cleavage of TNFR1 CTF abolished TNFR1 ICD formation while increasing TNFR1 CTF levels. Densitometry analysis of TNFR1 CTF levels also confirmed that it responded to DAPT as seen by increased immunoreactivity of the TNFR1 CTF **(Figure 4.2b)**. Next we examined whether generation of TNFR1 CTF and ICD were stimulated by TNFR1 ligand TNF α **(Figure 4.2c)**. Stimulation of HEK293T cells expressing TNFR1 with TNF α led to increased accumulation of TNFR1 ICD, and pre-treatment with DAPT inhibited constitutive and TNF α -stimulated TNF-R1 ICD fragment formation **(Figure 4.2c lane 3 & 4)**. The responsiveness to PMA and DAPT treatment was confirmed when we analyzed the cleavage of IL-1R1, a known γ -secretase substrate **(Figure 4.2d)**. Together this pharmacological data demonstrates that TNFR1 is a substrate for γ -secretase-mediated regulated intramembrane proteolysis.

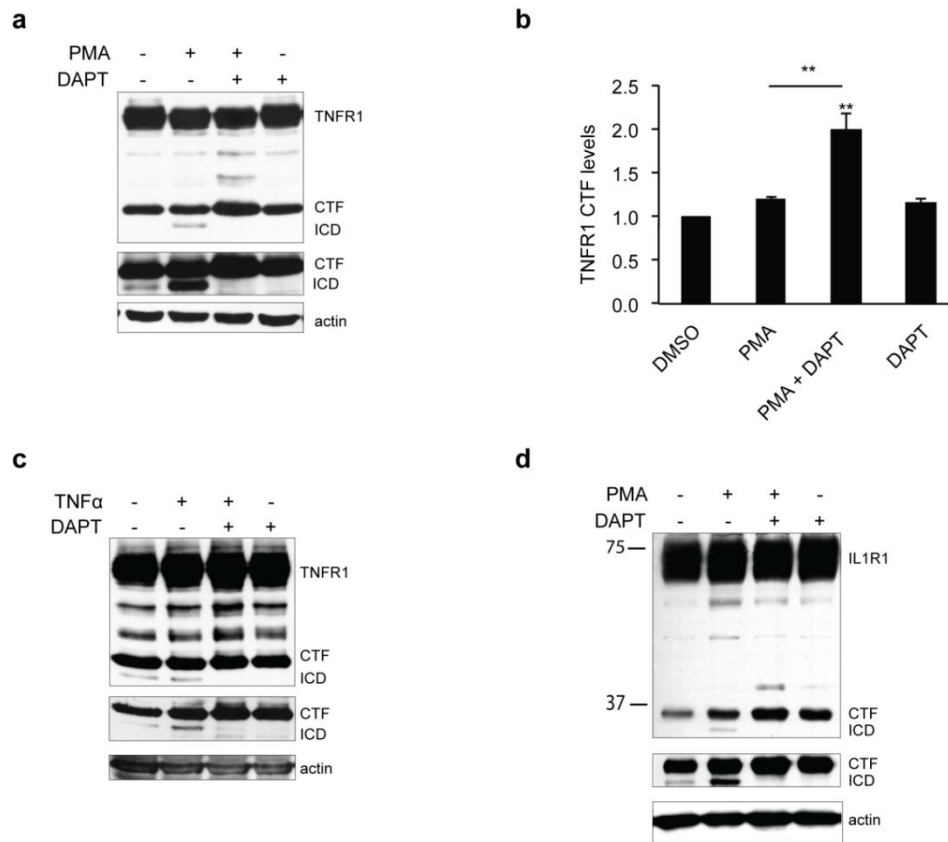


Figure 4.2 Ectodomain shedding-derived TNFR1 CTF is a substrate for γ -secretase

(a), (c) Immunoblot of cell lysates from HEK293T cells transiently transfected with TNFR1 and treated as indicated. C-terminus specific anti-TNFR1 antibody was used to assess cleavage profile of the receptor. **(b)** Densitometry analysis of TNFR1 CTF normalised to total β -actin for all experimental conditions. The amount of TNFR1 CTF is expressed as TNFR1 CTF immunoreactivity \pm standard error of the mean, representative of four independent experiments. Significance was calculated using two-way ANOVA, indicated with lines between the two compared groups. Asterisks without line indicate significance to the control- DMSO. **(d)** Immunoblot of cell lysates from HEK293T cells transiently transfected with IL1-R1, and treated with the same pharmacological modulators as TNFR1. C-terminus specific anti-IL-1R1 antibody was used to assess the cleavage profile for IL-1R1. The data is representative of four independent experiments.

4.2.2.2 PS-deficiency prevents γ -secretase-mediated proteolysis of TNFR1

PS proteins are necessary and required for γ -secretase activity. We next analyzed the cleavage of TNFR1 in wild-type (WT) and PS double-knock-out (PSDKO) murine embryonic fibroblasts (MEFs), which are deficient in PS-dependent γ -secretase activity. Immunoblot analysis of TNFR1 cleavage in untreated and PMA-stimulated cells revealed that wild-type MEFs over-expressing TNFR1 undergo constitutive and PMA-stimulated TNFR1 ICD formation (**Figure 4.3 lane 1 & 2**). In contrast, and as predicted, in PSDKO MEFs, the constitutive and PMA-stimulated generation of TNFR1 ICD was abolished, and a simultaneous accumulation of the CTF fragment was observed, indicative of a complete lack of γ -secretase activity (**Figure 4.3 lane 3 & 4**).

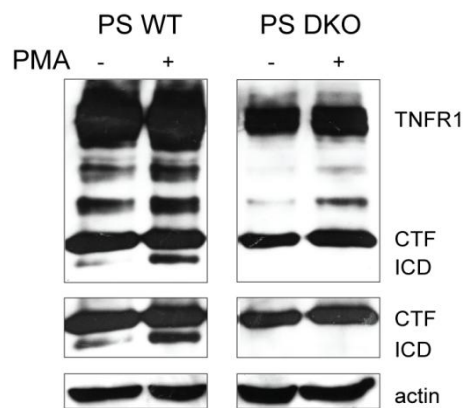


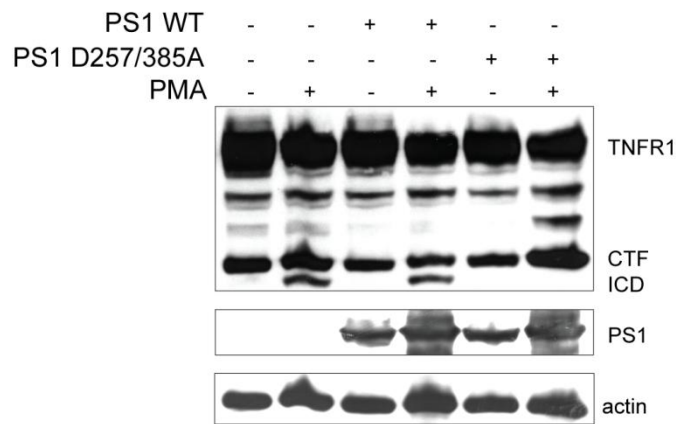
Figure 4.3 PS deficiency prevents γ -secretase-mediated regulated intramembrane proteolysis of TNFR1

Immunoblot of PS WT and PSDKO MEFs transiently expressing TNFR1 and probed with C-terminus specific anti-TNFR1 antibody, to allow assessment of its cleavage pattern following PMA treatment. The data is representative of three independent experiments.

4.2.3 PS1-dependent γ -secretase activity is required for cleavage of TNFR1 CTF

After confirming TNFR1 to be a novel γ -secretase substrate, we next used a genetic model of γ -secretase inactivation to determine whether it is cleaved by a PS1-containing or PS2-containing γ -secretase complex. The γ -secretase enzyme activity is attributed to two highly conserved aspartate residues in the transmembrane domain 6 and 7 of PS1 (PS1D257 and PS2D385) and PS2 (PS2D263 and PS2D366) (M S Wolfe et al., 1999). We analysed the cleavage of TNFR1 in HEK293T cells expressing TNFR1 and co-expressing either wild-type (WT) or dominant negative-PS1 (PS1^{D257A/D385A}) mutant and wild-type (WT) or dominant negative-PS2 (PS2^{D263A/D366A}) mutant. Immunoblot analysis of TNFR1 cleavage in cells treated with PMA revealed that cells expressing PS1 WT had no effect on γ -secretase mediated cleavage of TNFR1 (**Figure 4.4a lane 2 & 4**), but expression of the PS1^{D257A/D385A} mutant inhibited formation of the TNFR1 ICD and caused an accumulation of TNFR1 CTF (**Figure 4.4a lane 2 & 6**). In contrast, in HEK293T cells expressing TNFR1 and co-transfected with PS2 WT or PS2^{D263A/D366A} mutant, expression of PS2^{D263A/D366A} mutant had no effect on either the constitutive or PMA-induced generation of TNFR1 ICD (**Figure 4.4b lane 5 & 6**). This data suggests that like many other substrates (B De Strooper et al., 1998, 1999; Elzinga et al., 2009), TNFR1 is a substrate for PS1-dependent γ -secretase cleavage and is not cleaved by PS2 containing γ -secretase protease complex.

a



b

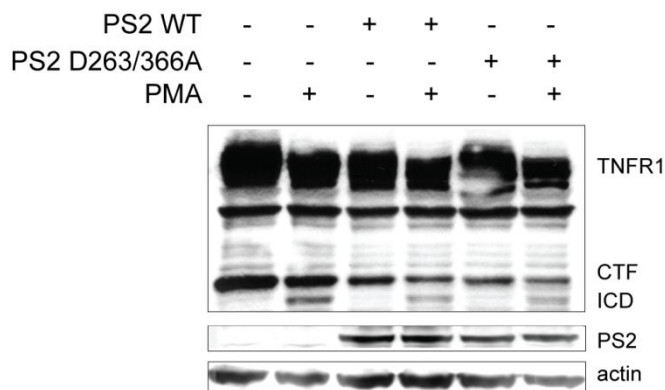


Figure 4.4 γ -secretase inactive PS1^{D257A/D385A} inhibits γ -secretase-dependent regulated intramembrane proteolysis of TNFR1

(a) Immunoblot of HEK293T cells transiently transfected with TNFR1 and co-transfected with either PS1 WT or PS1^{D257A/D385A} mutant. **(b)** Immunoblots of HEK293T cells transiently transfected with TNFR1 and co-transfected with either PS2 WT or PS2^{D263A/D366A} mutant. At thirty four hours post-transfection cells were treated with PMA (200ng/ml; 2h) to promote ectodomain shedding and subsequent γ -secretase-mediated cleavage. C-terminus specific anti-TNFR1 antibody was used to assess the cleavage profile of TNFR1. The data is representative of three independent experiments.

4.2.4 Ectodomain shedding is a pre-requisite for γ -secretase cleavage of TNFR1

It is reported that all type I transmembrane receptors which are subjected to γ -secretase-mediated cleavage, initially undergo ectodomain shedding in the extracellular domain as a pre-requisite to γ -secretase-mediated cleavage (Haapasalo et al., 2012). Next, using a TACE specific hydroxamate based metalloprotease inhibitor-TAPI; the importance of TACE-mediated ectodomain shedding of TNFR1 in γ -secretase-mediated cleavage was studied.

HEK293T cells were transiently transfected with TNFR1 and thirty-four hours post-transfection, cells were pre-treated with TAPI (10 μ M/ml; 2 h) in serum-free media, and subsequently treated with PMA (200ng/ml; 2h) to induce ectodomain shedding of TNFR1. Release of soluble TNFR1 (sTNFR1) ectodomain was clearly detectable in conditioned media of cells expressing TNFR1 as measured by ELISA (Invitrogen). TNFR1 underwent constitutive and PMA-induced ectodomain shedding, and PMA treatment led to a significant increase in the generation sTNFR1, which was inhibited by TAPI (**Figure 4.5a**). Immunoblot analysis of the same cultures with an anti-TNFR1 C25 antibody revealed that treatment with PMA also led to an increase in the generation of TNFR1 CTF and TNFR1 ICD (**Figure 4.5b**); suggesting ectodomain shedding is a prerequisite for γ -secretase-mediated cleavage of TNFR1.

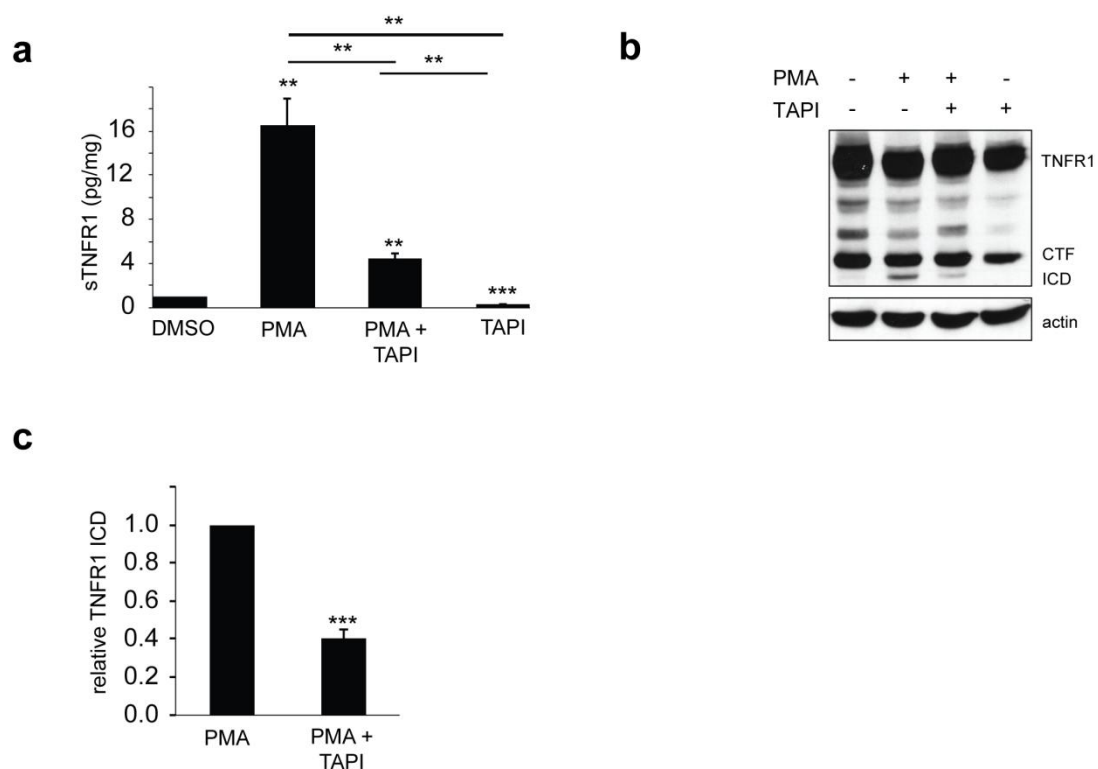


Figure 4.5 TAPI prevents ectodomain shedding and subsequent γ -secretase-mediated cleavage of TNFR1

(a) Analysis of sTNFR1 using conditioned media from cell plates treated with PMA to induce ectodomain shedding in presence or absence of TAPI and analysed using sTNFR1 specific ELISA (Invitrogen). Data expressed as picogram of sTNFR1 per milligram of total protein \pm standard error of the mean, representative of three independent experiments versus TNFR1 control. Significance was calculated using two-way ANOVA, indicated with lines between the two groups. Asterisks without line indicate significance to the control-DMSO. **(b)** Immunoblot analysis of corresponding cell lysates probed with C-terminus specific anti-TNFR1 antibody **(c)** Densitometry analysis of TNFR1 ICD normalised to total β -actin for all experimental conditions. The amount of TNFR1 ICD is expressed as TNFR1 ICD immunoreactivity \pm standard error of the mean, representative of three independent experiments. The data is representative of three independent experiments.

4.2.5 Inhibiting TNFR1 CTF internalization disrupts its γ -secretase-mediated cleavage

Once TNFR1 was identified as a new γ -secretase substrate, the subcellular occurrence of this cleavage event was studied. TNFR1 undergoes clathrin-mediated internalization (Schneider-Brachert et al., 2004; Stefan Schütze et al., 2008) Three different approaches were used to determine the effect of inhibiting clathrin-mediated internalization on γ -secretase-mediated cleavage of TNFR1.

4.2.5.1 Pharmacological inhibition of internalization

HEK293T cells were transiently transfected with TNFR1 and thirty-four hours post-transfection cells were pre-treated with dynasore (50 μ M/ml; 2h), a pharmacological inhibitor of clathrin-mediated endocytosis, or DMSO as a vehicle control in serum-free media followed by PMA (200ng/ml; 2h) treatment. ELISA analysis revealed that sTNFR1 was readily detected in conditioned media of cells treated with PMA, and a significant increase in sTNFR1 levels was seen following inhibition of TNFR1 internalization on treatment with dynasore (**Figure 4.6b**). Immunoblot analysis of corresponding cell lysates confirmed PMA-induced TNFR1 CTF and TNFR1 ICD generation and that treatment with dynasore, decreased formation of the TNFR1 ICD (**Figure 4.6a lane 3**). It is known that following TNF α binding, signalling from TNFR1 leads to the activation of both NF- κ B and the JNK pathways, where the TNFR1-mediated activation of NF- κ B is initiated at the plasma membrane, while activation of JNK is initiated following TNFR1 internalization (Schütze et al., 1999). To verify dynasore was indeed inhibiting TNFR1 internalization and affecting TNFR1 signalling events, wild-type MEFs were pre-treated with dynasore (50 μ M/ml; 2h) and stimulated with recombinant murine TNF α (mTNF α) for increasing times. Immunoblot analysis revealed that dynasore had no effect on TNF α -induced phosphorylation and degradation of the NF- κ B inhibitor, I κ B α . However, in the presence of dynasore, reduced phosphorylation and activation of JNK was

observed (**Figure 4.6c**). This demonstrates the functionality of dynasore in our experimental system as an inhibitor of TNFR1 internalization and TNF α -mediated signalling, and that inhibition of TNFR1 internalization reduced TNFR1 cleavage by γ -secretase.

4.2.5.2 Dynamin K44A mutant inhibits γ -secretase-mediated cleavage of TNFR1

Next a genetic approach was employed using wild-type dynamin (Dyn WT) or dynamin K44A mutant (Dyn-DN) which prevents clathrin-dependent internalization. HEK293T cells were transfected with TNFR1 and co-transfected with either Dyn WT or Dyn-DN. Immunoblot analysis revealed that while co-expression of Dyn WT had no effect on TNFR1 CTF or TNFR1 ICD formation, the co-expression of Dyn-DN had no effect on PMA-induced TNFR1 CTF formation but significantly reduced PMA-stimulated generation of TNFR1 ICD (**Figure 4.7a lane 4**). Simultaneous analysis of conditioned media from cells expressing either Dyn WT or Dyn-DN confirmed that inhibition of TNFR1 internalization had no effect on constitutive or PMA-induced ectodomain shedding and generation of sTNFR1 (**Figure 4.7b**). The functionality of the Dyn-DN as an inhibitor of TNFR1 internalization and signalling was confirmed by immunoblot analysis of TNFR1 CTF level in cytosolic fractions of HEK293T cells expressing TNFR1 and co-transfected with either Dyn WT or Dyn-DN. The cytosolic levels of TNFR1 CTF were found to increase on PMA treatment in the cytosolic fractions of the cells expressing Dyn WT but failed to do so in cells expressing Dyn-DN (**Figure 4.7c compare lane 2 and 6**). This indicates that while ectodomain shedding of TNFR1 occurs on the plasma membrane, internalization of the TNFR1 CTF is required for subsequent cleavage by γ -secretase. Again, as for our studies employing dynamin, to verify that Dyn-DN was inhibiting TNFR1 internalisation and affecting TNFR1 signalling events, wild-type MEFs co-expressing either Dyn WT or Dyn-DN were stimulated with mTNF α (**Figure 4.7d**). Immunoblot analysis revealed that Dyn WT had no effect on TNF α -induced phosphorylation and degradation of the NF- κ B inhibitor I κ B α . However, in cells expressing Dyn-DN reduced JNK activation was observed (**Figure 4.7d**). This validates our experimental system, and provides further evidence that inhibition of TNFR1 internalisation reduces TNFR1 cleavage by γ -secretase.

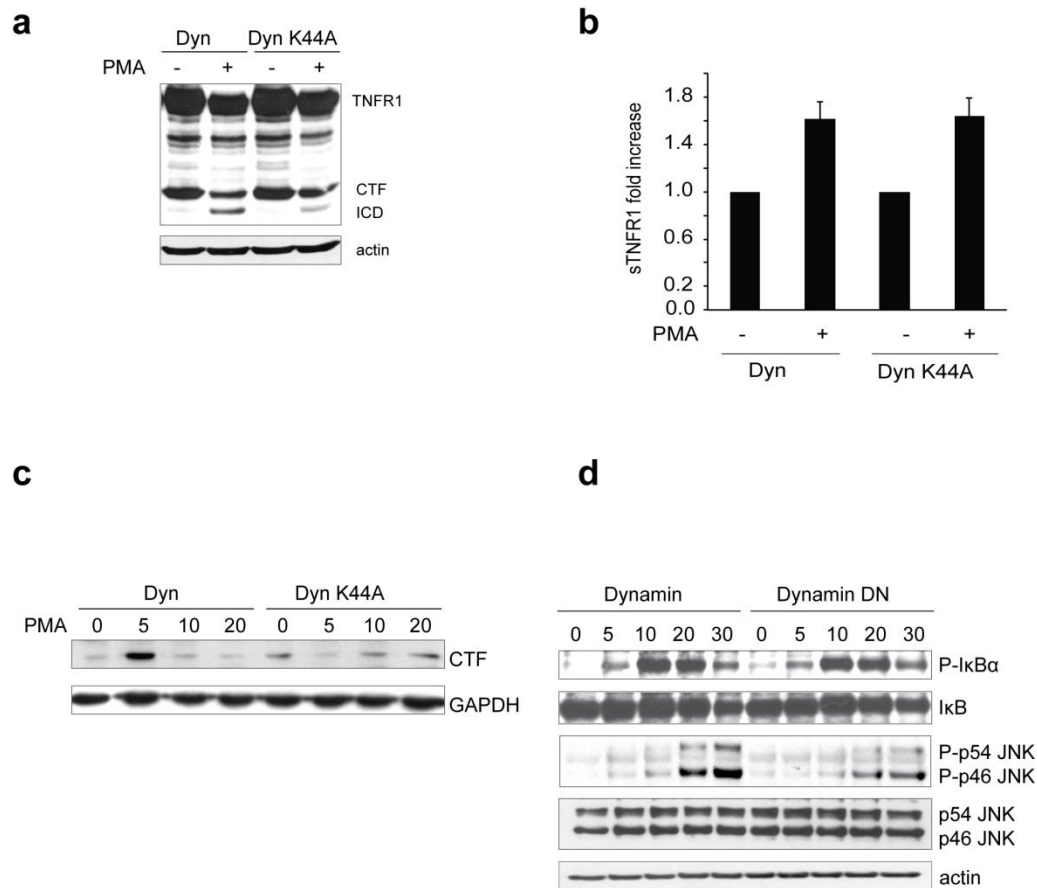


Figure 4.7 Over-expression of Dyn-DN prevents γ -secretase-mediated cleavage of TNFR1

(a) Immunoblot analysis of HEK293T cell lysates, transiently expressing TNFR1 and Dyn WT or Dyn-DN and stimulated with PMA. **(b)** Analysis of sTNFR1 from conditioned media from cell cultures treated with PMA to induce ectodomain shedding and analysed using sTNFR1 specific ELISA (Invitrogen). Data expressed as picogram of sTNFR1 per milligram of total protein \pm standard error of the mean, representative of three independent experiments versus TNFR1 control. (NSD) **(c)** Cytosolic fractions of HEK293T cells treated with PMA for increasing time and analysed for TNFR1 CTF. **(d)** Immunoblot analysis of cell lysates from MEF PS WT cells transiently transfected with Dyn WT or Dyn-DN, and stimulated with mTNF α for indicated times. The data is representative of four independent experiments.

4.2.5.3 TNFR1 internalization domain mutant does not undergo γ -secretase-mediated cleavage

Schneider-Brachert *et al.* identified a motif within the C-terminus domain of TNFR1 that mediated TNFR1 internalization known as the TNFR1 internalization domain or TRID. Deletion of this region failed to induce receptor endocytosis, subsequent formation of complex II and induction of apoptosis-inducing pathways. The authors also identified a highly conserved motif within TRID region, known to be involved in clathrin-mediated internalization of a number of cell surface receptors-YXXW sequence, comprised of tyrosine (Y) and tryptophan (W) enclosing any two amino acids. Mutations in this sequence caused TNFR1 to remain at the cell surface, essentially preventing internalization (Schneider-Brachert *et al.*, 2004).

TNFR1 TRID mutant at W239A, was made and employed in the study to determine the subcellular occurrence of the γ -secretase-mediated TNFR1 cleavage (**Figure 4.8a**). HEK293T cells were transfected with either TNFR1 or TNFR1 W239A and thirty-four hours post-transfection, cells were treated with PMA (200ng/ml; 2h) and lysed to assess the effect of internalization on γ -secretase-mediated cleavage. In cells expressing wild-type TNFR1, PMA-induced an increase in generation of TNFR1 CTF and TNFR1 ICD, however in cells expressing TNFR1 W239A mutant, formation of TNFR1 ICD was completely inhibited. Thus, inhibiting TNFR1 internalization clearly inhibited γ -secretase-mediated cleavage of the receptor (**Figure 4.8b lane 4**). Analysis of sTNFR1 levels in conditioned media from the same cell cultures confirmed that PMA-induced ectodomain shedding was not perturbed in cells expressing TNFR1 W239A (**Figure 4.8c**). However, increased levels of sTNFR1 were detected in cells over-expressing TNFR1 W239A which was attributed to increased localization of the receptor at plasma membrane. To validate TNFR1 W239A did not undergo internalization in the cell system used; cytosolic fractions were analyzed and used to check for internalization of

TNFR1 CTF following PMA-induced ectodomain shedding. TNFR1 W239A CTF was not detected to the same levels as in cells expressing TNFR1 WT, indicating that TNFR1 W239A did not undergo internalization (**Figure 4.8d**). Again, TNF α -mediated signalling and activation of both NF- κ B and JNK pathway was also examined. Wild-type MEFs expressing TNFR1 WT or TNFR1 W239A mutant were stimulated with mTNF α for the indicated times. Again, inhibition of TNFR1 receptor internalisation had no effect on TNF α -induced NF- κ B activation however; upon expression of TNFR1 W239A mutant reduced JNK activation was observed (**Figure 4.8e**). Collectively, this data demonstrates that ectodomain shedding of TNFR1 is a prerequisite for γ -secretase-mediated cleavage of TNFR1 and that γ -secretase-mediated cleavage of TNFR1 occurs following TNFR1 internalization.

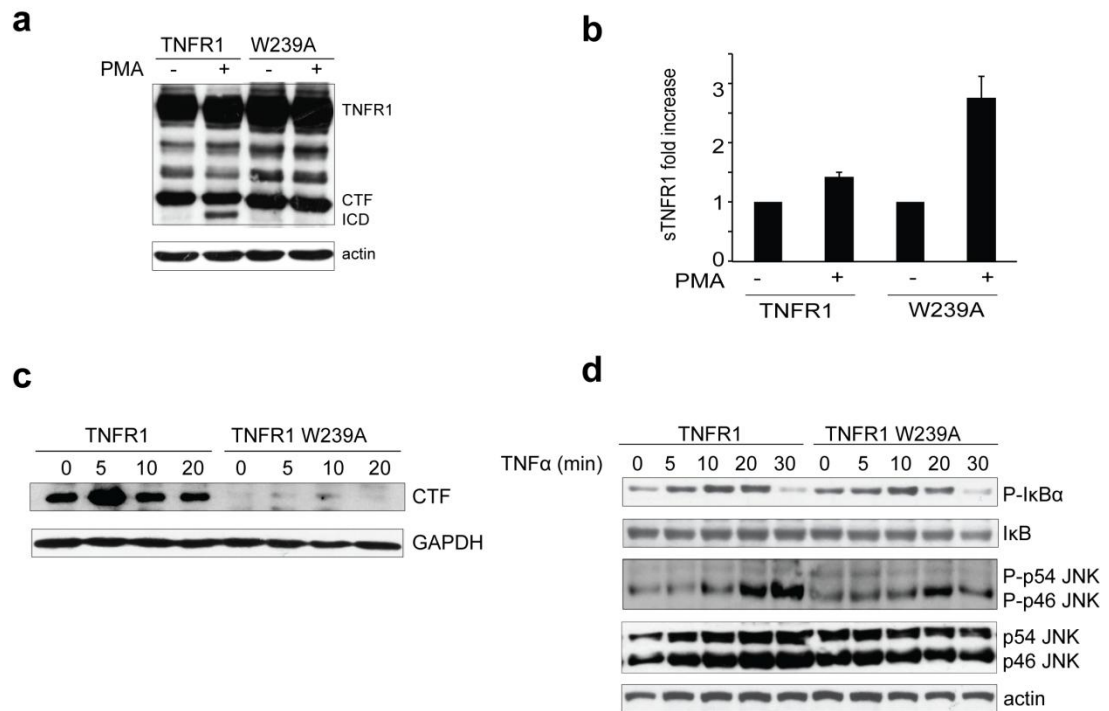


Figure 4.8 TNFR1 W239A mutant does not undergo γ -secretase-mediated cleavage

(a) Immunoblot analysis of HEK293T cells transiently transfected with TNFR1 and TNFR1 W239A treated with PMA (200ng/ml; 2h). **(b)** Analysis of sTNFR1 from conditioned media from cell cultures treated with PMA to induce ectodomain shedding and analysed using sTNFR1 specific ELISA (Invitrogen). Data expressed as picogram of sTNFR1 per milligram of total protein \pm standard error of the mean, representative of three independent experiments versus TNFR1 control. (NSD) **(c)** Cytosolic fractions of HEK293T cells treated for increasing time with PMA and analysed for TNFR1 CTF. **(d)** Immunoblot analysis of cell lysates from MEF PS WT cells transfected with either TNFR1 WT or TNFR1 W239A and stimulated with mTNF α for indicated time points. The data is representative of three independent experiments.

4.2.6 TNFR1 is cleaved by γ -secretase in early endosomes

To complement data obtained from inhibiting receptor internalization, we next examined the subcellular location of γ -secretase-mediated cleavage of TNFR1 was further examined using cells expressing Rab dominant negative (DN) mutants. Rab5 and Rab7 proteins belong to a superfamily of small molecular weight GTPases known to be associated with early and late endosomes, respectively. The Rab5 protein plays an important regulatory role in early endocytosis and trafficking to the early endosomes, while Rab7 plays a key role downstream of Rab5, in regulating membrane transport leading from early to late endosomes (Stenmark & Olkkonen, 2001).

4.2.6.1 Rab5-DN inhibits γ -secretase-mediated cleavage of TNFR1

HEK293T cells expressing TNFR1 were co-transfected with either Rab5 WT or Rab5-DN constructs. At thirty-four hours post-transfection cells were treated with PMA (200ng/ml; 1h) to promote ectodomain shedding and subsequent formation of TNFR1 ICD. Immunoblot analysis revealed that preventing trafficking of TNFR1 to the early endosome using Rab5-DN inhibited γ -secretase-mediated cleavage of the TNFR1 CTF, leading to decreased TNFR1 ICD formation (**Figure 4.9a lane 3 & 4**). On the other hand, Rab5 WT did not affect γ -secretase-mediated cleavage of TNFR1 and the TNFR1 ICD formation (**Figure 4.9a lane 1 & 2**). Next, subcellular fractionation was performed to check for the efficacy of the Rab5 constructs. In cells expressing Rab5 WT, TNFR1 receptor co-localized with the early endosomes, but decreased co-localization of TNFR1 and the early endosomal marker-EEA1 was observed in cells over-expressing Rab5-DN (**Figure 4.9b**). This data demonstrates that preventing the trafficking of TNFR1 to early endosomes negatively affects the γ -secretase-mediated cleavage of TNFR1.

4.2.6.2 Rab7-DN leads to an accumulation of TNFR1 CTF in early endosome, and increases generation of TNFR1 ICD

HEK293T cells expressing TNFR1 were co-transfected with either Rab7 WT or Rab7-DN constructs. At thirty-four hours post-transfection cells were treated with PMA (200ng/ml; 90min) to promote ectodomain shedding and subsequent formation of TNFR1 ICD. Immunoblot analysis revealed that preventing trafficking of TNFR1 from the early endosome to late endosome using Rab7-DN enhanced TNFR1 ICD formation (**Figure 4.10b lane 3 & 4**). On the other hand Rab7 WT construct did not significantly affect γ -secretase-mediated cleavage of TNFR1 and the TNFR1 ICD formation (**Figure 4.10b lane 1 & 2**). Subcellular fractionation was performed to check for the efficacy of the Rab7 constructs. Immunoblot analysis of cytosolic fractions revealed that in cells expressing Rab7 WT, TNFR1 CTF co-localized in the fractions enriched with the early endosomes. In contrast, co-localization of both TNFR1 CTF and TNFR1 ICD with fractions positive for the early endosomal marker-EEA1 was observed in cells over-expressing Rab7-DN (**Figure 4.10c**). Thus demonstrating that the inhibition of TNFR1 trafficking from the early endosome increases γ -secretase-mediated cleavage of TNFR1. Collectively, this data demonstrates that TNFR1 undergoes γ -secretase-mediated cleavage in the early endosome.

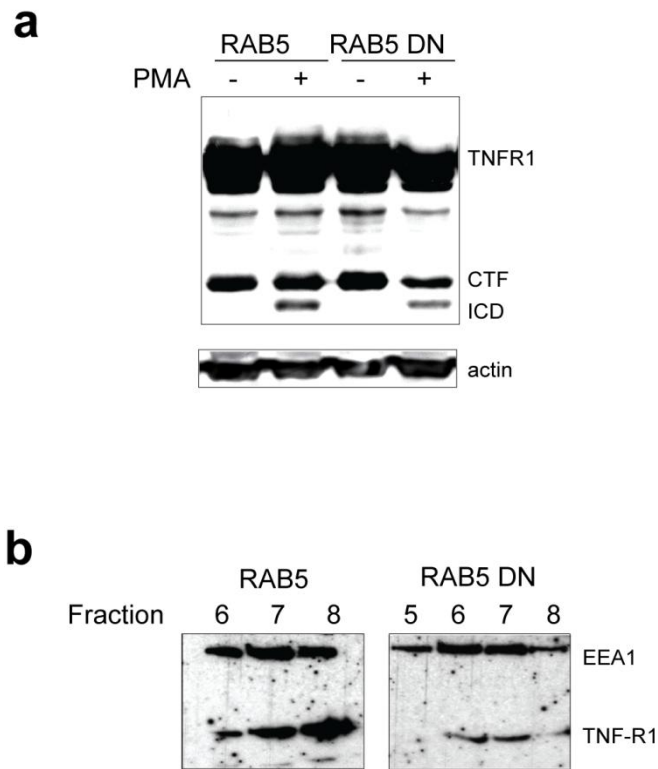


Figure 4.9 Preventing early endosome fusion reduces γ -secretase-mediated cleavage of TNFR1

(a) Immunoblot analysis of HEK293T cells expressing TNFR1 and co-transfected with either Rab5 WT or Rab5 DN construct and treated with PMA to induce ectodomain shedding **(b)** HEK293T cells transiently expressing TNFR1 and co-transfected with Rab5WT and Rab5 DN; treated with PMA and analysed for full length TNFR1 co-localizing with the early endosomal fraction. C-terminus specific anti-TNFR1 antibody was used to assess cleavage profile of TNFR1. The data is representative of three independent experiments.

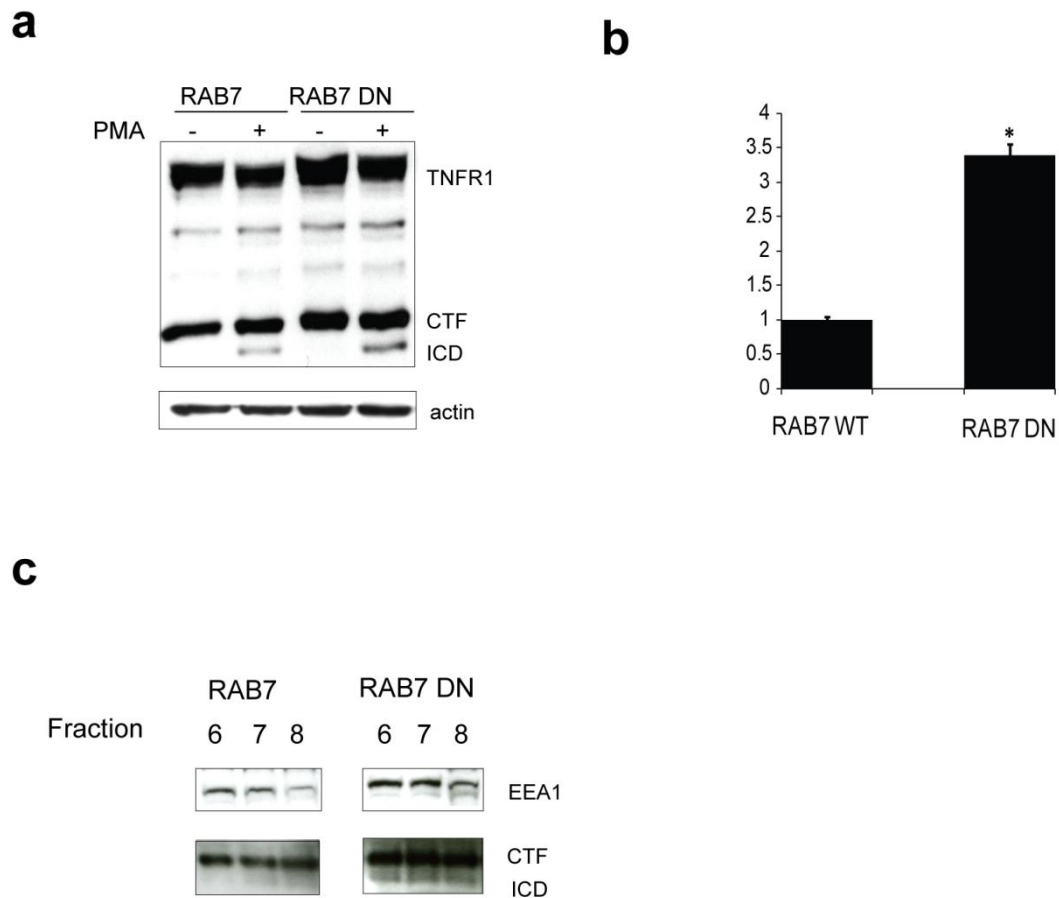


Figure 4.10 γ -secretase-mediated cleavage of TNFR1 occurs in the early endosome

(a) Immunoblot analysis of HEK293T cells expressing TNFR1 and co-transfected with either Rab7 WT or Rab7 DN construct and treated with PMA to induce ectodomain shedding **(b)** Densitometry analysis of TNFR1 ICD normalised to total β -actin for all experimental conditions. The amount of TNFR1 ICD is expressed as TNFR1 ICD immunoreactivity \pm standard error of the mean, representative of three independent experiments. **(c)** HEK293T cells transiently expressing TNFR1 and co-transfected with Rab7 WT and Rab7 DN; treated with PMA and analysed for full length TNFR1 co-localizing with the early endosomal fraction. C-terminus specific anti-TNFR1 antibody was used to assess cleavage profile of TNFR1. The data is representative of three independent experiments.

4.3 Discussion

4.3.1 Identifying TNFR1 as a novel γ -secretase substrate

The PS proteins are involved in regulating the responsiveness of neuronal cells to apoptosis (Bulat et al., 2009). As previously described, PS proteins are the catalytic components of γ -secretase enzyme complex with over 91 substrates. However, cell death receptors as potential substrates for γ -secretase mediated cleavage have not been studied. We screened the TNFR superfamily members: TNFR1, TNF-R2, DR3 and DR4 as potential substrates. Similar to p75NTR, IL-1R1 and Notch (Elzinga et al., 2009; Levine et al., 1996; Weskamp et al., 2004); TNFR1, TNF-R2, DR3 and DR4 are type 1 transmembrane proteins, therefore fitting the criteria of previously identified γ -secretase substrates. Furthermore, TNFR1 and TNF-R2 undergo ectodomain shedding, which is considered to be a pre-requisite for subsequent γ -secretase-mediated cleavage. In this context, TNFR1 was found to be cleaved by γ -secretase enzyme complex.

PMA treatment of cells expressing TNFR1 led to the formation of a fragment of ~38-kDa and a smaller fragment of ~32-kDa. This was consistent with the cleavage profile of previously established γ -secretase substrates such as IL-1R1, Notch, p75, APP, Mucin etc. (Haapasalo et al., 2012). This observation prompted us to investigate whether the TNFR-1 underwent γ -secretase-mediated cleavage following ectodomain shedding. We employed both pharmacological and genetic approaches. Consistent with previous studies (Becker-Herman et al., 2005; Böhm et al., 2006; Elzinga et al., 2009), DAPT alone or in combination with PMA inhibited the formation of TNFR1 ICD with a simultaneous accumulation of the CTF (**Figure 4.2a**). Furthermore ligand induced ICD formation was also inhibited by DAPT (**Figure 4.2c**). PS proteins are required for γ -secretase activity. Thus, MEF PSDKO cells devoid of γ -secretase activity were used. Immunoblot analysis revealed that MEF PSDKO cells were defective in ICD formation

(Figure 4.3). Thus we showed that TNFR1 undergoes PS-dependent γ -secretase-mediated cleavage.

Considering the distinct roles played by the two PS proteins in apoptosis, the finding that TNFR1 cleavage was a γ -secretase substrate was very intriguing. For instance, previous studies have demonstrated that loss or down-regulation of PS1 expression is associated with a loss of neuronal differentiation and increased apoptotic cell death (Roperch et al., 1999; Weihl et al., 1999; Maraver et al., 2007). Thus, indicating a pro-apoptotic role of PS1 protein. In contrast the wild-type and FAD-linked PS2 has been shown to trigger apoptosis and down-regulate PS1 expression through p53-dependent mechanisms (Smith et al., 2002). Collectively, these findings point towards the role played by PS proteins in regulating apoptosis. The γ -secretase enzyme complex consists of four different subunits: NCT, Aph-1a or Aph-1b, Pen-2, PS1 or PS2 and depending upon the subunit involved, four different γ -secretase complexes can co-exist. Once PS-dependent γ -secretase-mediated cleavage of TNFR1 was confirmed, the next question that arose was whether PS1 or PS2-mediated this cleavage event. To answer this question, γ -secretase inactive PS1 and PS2 mutants were used. TNFR1 was co-expressed with either PS1 WT or γ -secretase inactive PS1^{D257/385A} **(Figure 4.4a)**. The use of catalytically inactive PS1 did raise the possibility that PS1^{D257/385A} over-expression led to increased binding of PS1 WT or mutant, containing γ -secretase enzyme complex to TNFR1 CTF, making it unavailable to PS2 containing γ -secretase complex. Hence, to rule out competitive inhibition we also used PS2 WT and PS2^{D263/366A} to determine the effect of lack PS2-dependent γ -secretase activity **(Figure 4.4b)**. The results clearly showed that TNFR1 γ -cleavage was dependent upon the PS1-mediated γ -secretase activity and independent of PS2.

4.3.2 Subcellular location for shedding and γ -secretase cleavage of TNFR1

Ectodomain shedding is a characteristic of most known γ -secretase substrates. To determine whether ectodomain shedding of TNFR1 and γ -secretase-mediated cleavage of CTF were sequential events; a pharmacological inhibitor of TACE-mediated cleavage-TAPI was used. Inhibiting ectodomain shedding of TNFR1 resulted in decreased ICD formation, clearly indicating that ectodomain shedding of TNFR1 was a pre-requisite to γ -secretase-mediated cleavage of TNFR1 CTF (**Figure 4.5**).

The TNF super-family of ligand and their receptors initiate an array of signalling pathways, leading to diverse cellular responses such as cell survival or death. TNF α -signalling is preferentially mediated through TNFR1. Following ligand binding, TNFR1 recruits the adaptor protein TRADD via the homotypic DD interactions. TRADD then recruits TRAF2, RIP1 and other associated proteins-UBC13, cIAP1/2 (Zarnegar et al., 2008). The complex thus formed at the cell surface is referred to as the Complex I which signals for the NF- κ B activation. The TNFR1 complex I then undergoes internalization and gets dissociated. This event leads to the formation of endosomal complex II, thereby initiating the pro-apoptotic signalling. The compartmentalization of TNFR1 mediated signalling acts as a switch between the anti- and pro-apoptotic signals. Thus, we next tried to gain a better understanding of the role γ -secretase-mediated cleavage of TNFR1 might play in signalling pathway from Complex I and Complex II. Furthermore, due to the apparent discrepancy between the subcellular localization of PS proteins and cleavage of the known γ -secretase substrates an open question that arose was the subcellular site for the occurrence of γ -secretase-mediated cleavage of TNFR1 CTF.

To address this question- pharmacological and genetic approaches were used. TNFR1 is known to undergo clathrin mediated internalization (Mosselmans et al., 1988; Schütze et al., 1999). In a pilot experiment the pharmacological inhibitor of

internalization, dynasore, capable of inhibiting clathrin mediated internalization was used. Dynasore treatment led to increased retention of the receptor at the cell surface with a simultaneous increase in levels of sTNFR1 and decreased ICD formation (**Figure 4.6**). Lack of a TNFR1 C-terminus antibody for immunofluorescence restricted the study to validate the functionality of the inhibitor. Since, inhibiting TNFR1 internalization has been shown to have no effect on TNF α -induced NF- κ B activation but delay the JNK activation, we studied TNF α -induced NF- κ B and JNK activation (Schütze et al., 2008). Consistent with the study, inhibiting clathrin mediated TNFR1 internalization had no effect on TNF α -induced NF- κ B activation but delayed the JNK activation (**Figure 4.6c**).

Dynamin-DN, defective in clathrin mediated internalization was also used. Co-expression of dynamin-DN with TNFR1 led to increased ectodomain shedding of the receptor and decreased levels of TNFR1 ICD. Simultaneously, defective TNF α -induced JNK activation was observed in dynamin-DN expressing cells with no effect on NF- κ B signalling. Cytosolic fractions were isolated and levels of TNFR1 CTF internalized from the cell surface after PMA treatment were determined. Decreased CTF levels were detected in cells co-expressing TNFR1 and dynamin-DN cells (**Figure 4.7**).

One could argue that inhibiting clathrin mediated internalization affected the trafficking of the receptor; however it also altered the functionality of the γ -secretase enzyme by altering its trafficking and assembly. To rule out this possibility, TNFR1 internalization mutant was made. Schneider-Brachert *et al.* identified a highly conserved motif YXXW within TNFR1 internalization domain (TRID) region. The W residue was mutated in the receptor and TNFR1 W239A construct was used. TNFR1 W239A expressing cells showed no ICD formation after PMA treatment. Similar to dynasore treated and dynamin-DN expressing cells, increased ectodomain shedding was observed for TNFR1 W239A. Cytosolic fractions of TNFR1 W239A showed no change in levels of TNFR1 CTF on PMA treatment (**Figure 4.8**). Together this data

showed that inhibiting TNFR1 internalization disrupted γ -secretase-mediated cleavage of TNFR1.

TNFR1 is most extensively studied receptor of the TNF superfamily owing to its dual functionality. As previously mentioned it is capable of sequentially inducing both anti- and pro-apoptotic signals, signalling from cell surface and endosome respectively. This was intriguing, considering the γ -cleavage event occurred post internalization. In the next study, Rab5 and Rab7 constructs capable of specifically inhibiting the membrane trafficking of the receptor from cell surface to early endosome and to the late endosome were used. Rab5-DN prevented fusion of endocytic vesicles to form early endosome. Over-expression of Rab5-DN in HEK293T cells reduced TNFR1 ICD formation. To determine the efficacy of the mutant early endosomes were isolated by sucrose gradient fraction. Fractions enriched in early endosomes were analysed for TNFR1. Decreased levels of TNFR1 were detected in cells co-expressing Rab5-DN mutant (**Figure 4.9**). In parallel Rab7-DN construct which prevents trafficking of the receptor from early to late endosome was also used. Co-expression of TNFR1 with Rab7-DN led to accumulation of TNFR1 CTF in the early endosome as shown by sucrose gradient fractionation and isolation of early endosomal fraction. Simultaneously increased ICD was detected in these early endosomes, due to γ -secretase-mediated cleavage of the receptor. Whole cell lysates were analysed for TNFR1 cleavage and significant increase in ICD was detected (**Figure 4.10**). The data showed that γ -secretase-mediated cleavage of TNFR1 CTF occurred on the early endosomes.

4.3.2 Conclusion and future direction

In this study, TNFR1 is shown for the first time to undergo PS1-dependent γ -secretase-mediated cleavage. Additionally, ectodomain shedding of TNFR1 and its γ -secretase-mediated cleavage are shown to be two sequential cleavage events, where γ -secretase-mediated cleavage event occurs post internalization and before exit from

the early endosomes. The data extends the regulation of TNFR1 signalling and its compartmentalization to a next step, where γ -secretase enzyme complex might play an important role. Based on the data, the importance of PS1-dependent γ -secretase-mediated cleavage on regulating TNF α -induced signalling event was subsequently studied.

Chapter 5

Role of γ -secretase-mediated cleavage in spatial segregation and TNFR1 signalling

5.1 Introduction

Independent research groups have demonstrated by yeast-two-hybrid or co-immunoprecipitation approaches that PS proteins interact with a diverse array of proteins (Chen et al., 2002). In some cases, while the biological significance of the association with PS proteins is not apparently obvious, these proteins can be subdivided into functional groups including those involved in γ -secretase protein complex, Wnt signalling, cell-cell adhesion, vesicular transport, calcium signalling and apoptosis.

Our group previously reported and characterized of a highly conserved TRAF6 consensus-binding site within the hydrophilic loop domain of PS-1 (Elzinga et al., 2009), additionally TRAF6-mediated ubiquitination of IL-R1 was shown to enhance its regulated intramembrane proteolysis which in turn was mediated by PS1-dependent γ -secretase-mediated activity (Twomey et al., 2009). In another study we have demonstrated that PS1 interacts with TRAF2 (unpublished). TRAF2 also interacts with other TNF receptor superfamily members CD30 and CD40 via a major TRAF2-binding consensus sequence, (P/S/A/T)x(Q/E)E (Ye et al., 1999), or is recruited to TNFR1 through its association with the TRADD adaptor protein (Hsu et al., 1996). Therefore, the finding that TNFR1 also undergoes PS1-dependent γ -secretase-mediated cleavage prompted us to examine whether similar to TRAF6 and IL-1R1, TRAF2-induces ubiquitination of TNFR1 and plays a role in regulating the intramembrane proteolysis of TNFR1.

TRAF2 is also an important adaptor protein in TNF α -mediated TNFR1 signalling pathway (Devin et al., 2000; Hsu et al., 1996). Upon TNF α stimulation, TNFR1 complex I is formed at the cell surface which is important for cell survival and inflammatory signalling (Schneider-Brachert et al., 2004; Schütze et al., 2008; Tchikov et al., 2011). Several factors regulate the transition of Complex I to the cytosolic death-inducing

TNFR1 complex II, which triggers cell death. Recent reports have shown that the adaptor proteins recruited within this TNFR1 complex I i.e., RIP1, TRADD and TRAF2-regulate the formation of complex II and its output (Hsu et al., 1996; Kim et al., 2000). In addition to these three adaptor proteins; A20, an ubiquitin-editing enzyme, cIAP1, an ubiquitylating enzyme, LUBAC, a linear ubiquitylating enzyme complex, and TAK1 are also a part of complex I which negatively regulate the formation of complex II (Gyrd-Hansen et al., 2010; Haas et al., 2009; Walczak, 2011). There is also a second level of regulation by the activity of cylindromatosis (CYLD), a deubiquitylating enzyme which controls the transition from TNFR1 complex I to the cytosolic death-inducing TNFR1 complex II (Brummelkamp et al., 2003). Additionally, the composition of complex II determines the cell death outcome: apoptosis or necrosis. While TNFR1 signalling complex II containing TRADD associated with FADD, c-FLIP and caspase-8 induces apoptosis and suppresses the induction of necrosis; the association of RIP1 with FADD, c-FLIP and caspase-8 leads to the induction of necrosis **(Reviewed in Figure 12)**.

In this context, the finding that PS1-dependent γ -secretase-mediated cleavage of TNFR1 occurred post-internalization prompted us to explore the role played by PS1-dependent cleavage event in controlling the cell survival and cell death signals. The PS proteins are co-regulatory and have antagonistic functions. This is supported by several studies, for instance p53 inhibits expression of PS1, subsequently resulting in apoptosis and tumor suppression (Roperch et al., 1998), additionally over-expression of PS2, independent of its γ -secretase activity is also known to trigger p53-dependent apoptosis and simultaneously down regulate the expression of PS1 (Smith et al., 2002). Based on this information, we hypothesized a role for γ -secretase-mediated cleavage of TNFR1 on its signalling cascade.

The aims of the research presented in this chapter are

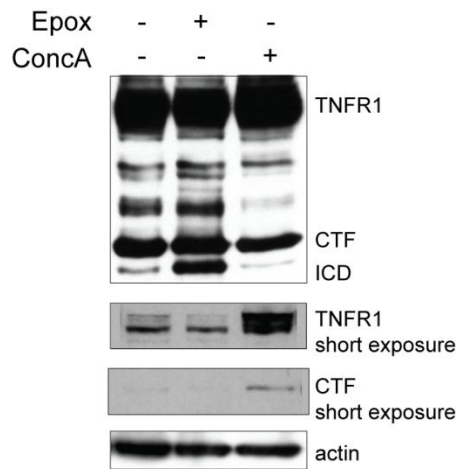
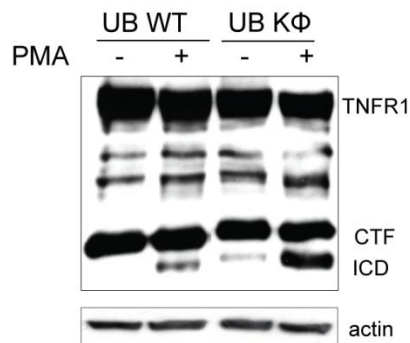
1. To study TRAF2-mediated ubiquitination of TNFR1 and its effect on regulated intramembrane proteolysis of TNFR1.
2. To determine the role played by PS proteins in TNF α -mediated signalling pathways.
3. To study the interaction between PS1 and the TNFR1 adaptor proteins-RIP and TRADD.

5.2 Results

5.2.1 TNFR1 ICD undergoes proteasomal degradation

After determining the subcellular location for γ -secretase-mediated cleavage of TNFR1, the fate of TNFR1 ICD was studied using epoxomicin, a lysosomal and concanamycin A, a proteasomal degradation inhibitor. HEK293T cells over-expressing TNFR1 were treated with either epoxomicin (10 μ M/ml; 2h) or concanamycin A (50 μ M/ml; 2h). Cells were harvested, lysed and the lysates were immunoblotted with C-terminus specific anti-TNFR1 antibody. Treatment with epoximicin increased detection of the TNFR1 ICD, where as TNFR1 full length and TNFR1 CTF accumulated in presence of concanamycin A. This data shows that TNFR1 ICD undergoes proteasomal degradation (**Figure 5.1a lane 1 & 2**), while full length TNFR1 and TNFR1 CTF are degraded via a lysosomal pathway (**Figure 5.1a lane 1 & 3**).

This finding was further validated using a Ubiquitin mutant in which all lysine residues were mutated (Ub K ϕ), and is therefore defective in the formation of polyubiquitin chains. HEK293T cells were transfected with TNFR1 and co-transfected with wild-type ubiquitin (Ub WT) or Ub K ϕ mutant and thirty-four hours post-transfection, cells were treated with PMA (200ng/ml; 2h). Immunoblot analysis of cell lysates revealed that PMA-induced the formation of TNFR1 CTF and ICD in Ub WT transfected cells (**Figure 5.1b lane 1 & 2**) while increased accumulation of TNFR1 ICD was observed in untreated and PMA-treated cells transfected with Ub K ϕ (**Figure 5.1b lane 3 & 4**). This data demonstrated that while polyubiquitination is not necessary for the formation of TNFR1 ICD, polyubiquitination of TNFR1 ICD is required for its degradation and not formation.

a**b****Figure 5.1 TNFR1 ICD undergoes proteasomal degradation**

(a) Immunoblot analysis of HEK293T cells transiently expressing TNFR1 and treated with proteasomal inhibitor-epoxomicin (10 μ M) and lysosomal (50 μ M) inhibitor-concanamycin for 2h. **(b)** HEK293T cells co-expressing TNFR1 and Ubiquitin WT or Ubiquitin K Φ ; treated with PMA (200ng/ml; 2h) and immunoblotted using C-terminus specific anti-TNFR1 antibody to assess the cleavage profile of the receptor. The data is representative of three independent experiments.

5.2.2 TRAF2 E3 ligase regulates constitutive and PMA-induced TNFR1 ICD formation

The above data shows that polyubiquitination is required for TNFR1 ICD degradation. Previous studies by our group showed TRAF6-mediated ubiquitination of IL-1R1 and subsequent enhanced regulated intramembrane proteolysis (Twomey et al., 2009). In this context the possibility of TRAF2 playing a role in ubiquitination of TNFR1 was explored. TRAF2 interacts with two members of the TNF receptor superfamily, CD30 and CD40, via a major TRAF2-binding consensus sequence, (P/S/A/T)x(Q/E)E (Ye et al., 1999). Additionally, upon TNF α binding TNFR1 recruits TRADD via the homotypic DD interactions, following which the N-terminal domain of TRADD interacts with TRAF2 (Hsu et al., 1996). Considering the redundancy amongst the TRAF family members, TRAF6 WT and TRAF6 DN lacking the E3 ligase activity was also included in this study. HEK293T cells transiently expressing TNFR1 and Ub K ϕ were co-transfected with TRAF2 WT or TRAF2 DN and TRAF6 WT or TRAF6 DN; and thirty-four hours post-transfection, cells were treated with PMA (200ng/ml; 2h). Immunoblot analysis revealed that the cells co-transfected with TRAF2 WT undergo constitutive and PMA-induced TNFR1 CTF and ICD formation (**Figure 5.2a lane 1 & 2**), while the cells co-expressing TRAF2 DN show reduced constitutive and PMA-induced TNFR1 ICD formation subsequent to ectodomain shedding (**Figure 5.2a lane 3 & 4**). In contrast, and consistent with a non-essential role for TRAF6 in TNFR1 signalling, co-expression of TRAF6 WT or TRAF6 DN, does not affect TNFR1 ICD formation (**Figure 5.2b lane 3 & 4**). Though not conclusive, this data demonstrates that E3 ligase activity of TRAF2 might participate in the regulated intramembrane proteolysis of TNFR1.

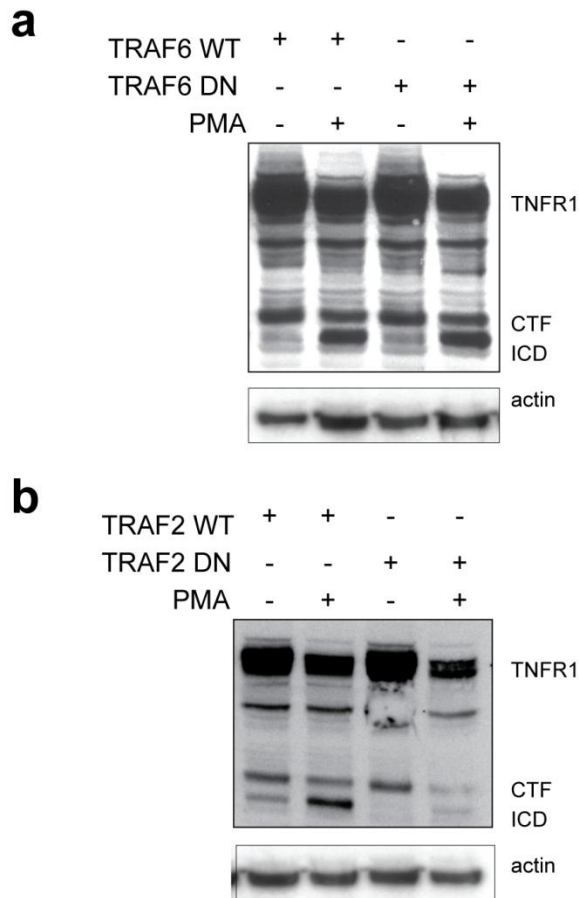


Figure 5.2 Dominant negative TRAF2 E3 ligase reduces constitutive and PMA-induced TNFR1 ICD formation

Immunoblot analysis of HEK293T cells transfected with expression vectors as indicated in the figure, thirty hours post-transfection cells were treated with PMA (200ng/ml; 2h), lysed and the blots were probed with C-terminus specific anti-TNFR1 antibody to assess the cleavage profile of the receptor. The data is representative of three independent experiments.

5.2.3 TNFR1 undergoes TRAF2-mediated polyubiquitination

Having shown that TRAF2-E3 ligase activity played a role in modulating TNFR1 ICD formation, the next question was whether TNFR1 undergoes TRAF2-mediated ubiquitination. To address this, HEK293T cells were transfected with TNFR1 and HA-Ub and then co-transfected with FLAG-TRAF2 WT or FLAG-TRAF2 DN as indicated (**Figure 5.3a**) and thirty-four hours post-transfection cells were lysed and immunoblotted with C-terminus specific anti-TNFR1 antibody and anti-FLAG antibody to confirm the expression of constructs and equivalency of loading (**Figure 5.3a, lower panels**). Similarly to distinguish whether TRAF2-induced mono- or polyubiquitination of TNFR1, we used HA-Ub K ϕ mutant and checked for equivalent expression of the constructs (**Figure 5.3b**).

After confirming the equivalent expression of the various plasmids, cells were lysed under stringent SDS-denaturing conditions. TNFR1 was immunoprecipitated with a C-terminus specific anti-TNFR1 antibody followed by western blotting with anti-HA or anti-TNFR1 antibody to detect ubiquitinated-TNFR1 or TNFR1 respectively. Immunoblot analysis revealed that in HA-Ub WT expressing cells, in the presence of TRAF2, TNFR1 undergoes ubiquitination (**Figure 5.3 a, upper panels lane 1 & 2**) while in the presence of TRAF2 DN, ubiquitination of TNFR1 is significantly reduced (**Figure 5.3 a, upper panels lane 3 & 4**), in contrast in cells expressing HA-Ub K ϕ , no difference is seen in cells expressing TRAF2 WT or TRAF2 DN, with or without PMA-treatment. This data demonstrates that TNFR1 undergoes TRAF2-mediated polyubiquitination.

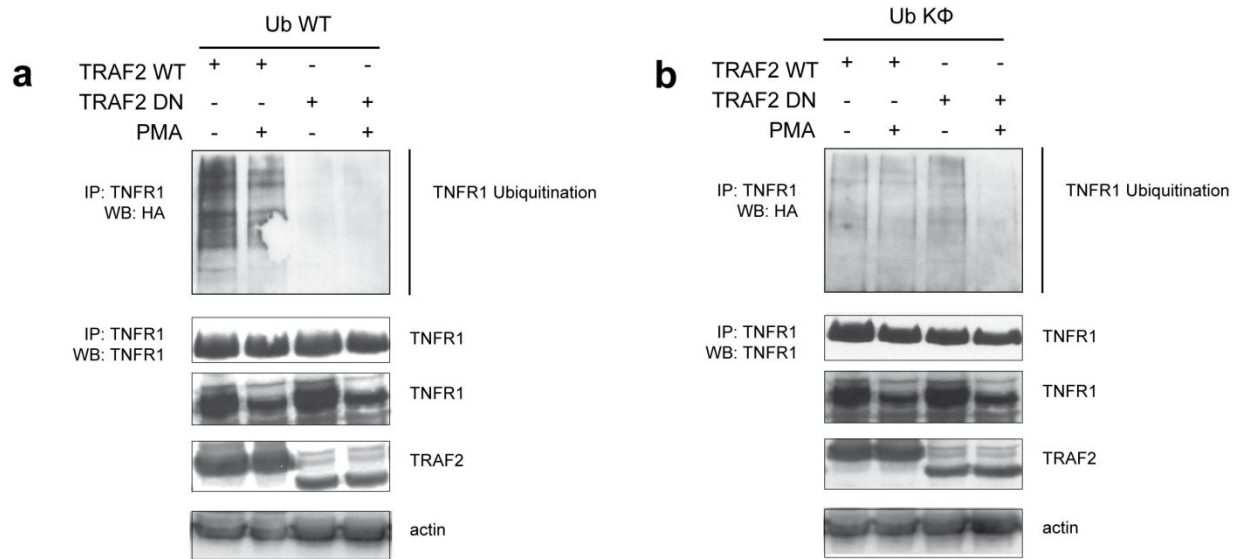


Figure 5.3 TNFR1 undergoes TRAF2-mediated polyubiquitination

(a) HEK293T cells were transiently expressing TNFR1 and HA-Ub were co-transfected with TRAF2WT or TRAF2 DN. **(b)** HEK293T cells were transiently expressing TNFR1 and HA-Ub K Φ were co-transfected with TRAF2WT or TRAF2 DN. Forty hours post-transfection cells were harvested under stringent SDS denaturing condition and then subjected to immunoprecipitation for TNFR1 and Western blot for HA-Ub. Ubiquitinated TNFR1 was detected with anti-HA antibody. Blot was also stripped and reprobed with an anti-TNFR1 antibody as a control. Lower panel shows the expression levels of the TNFR1, TRAF2 WT and TRAF2 DN in the HEK293T cell lysates used for ubiquitination. The data is representative of two different experiments.

5.2.4 Inhibiting γ -secretase-mediated cleavage of TNFR1 does not affect the TNF α -mediated signalling pathway

An important event in TNF α -mediated TNFR1 signalling is the activation of MAPK (JNK, p38 and ERK1/2), and canonical NF- κ B transcription factor from the cell surface (Verstrepen et al., 2008). To determine whether or not inhibition of γ -secretase activity and cleavage of TNFR1 had any effect on the activation of MAPK and NF- κ B, we next examined the transient activation of JNK1/2 and NF- κ B, in PS WT MEFs that were either untreated or treated with DAPT, and in PSDKO MEFs that are deficient in γ -secretase activity.

PS WT MEFs were serum starved overnight and pre-treated with DAPT (10 μ M/ml; 8h), following which cells were stimulated with TNF α (10ng/ml), for increasing time (**Figure 5.4a**). Alternately, PS WT and PS DKO MEFs cells were serum starved overnight and stimulated with TNF α (10ng/ml) for increasing time (**Figure 5.4b**). Western blot analysis was carried out to determine the effect of inhibiting γ -secretase activity on the activation of TNF α -induced signalling pathways. Immunoblot analysis revealed that inhibition of γ -secretase activity by using either γ -secretase inhibitor DAPT or PSDKO MEFs, does not affect the activation of JNK1/2 and NF- κ B. This data shows that activation of TNF α -mediated signalling is independent of its cleavage by γ -secretase and that γ -secretase activity is not essential for TNFR1 signalling.

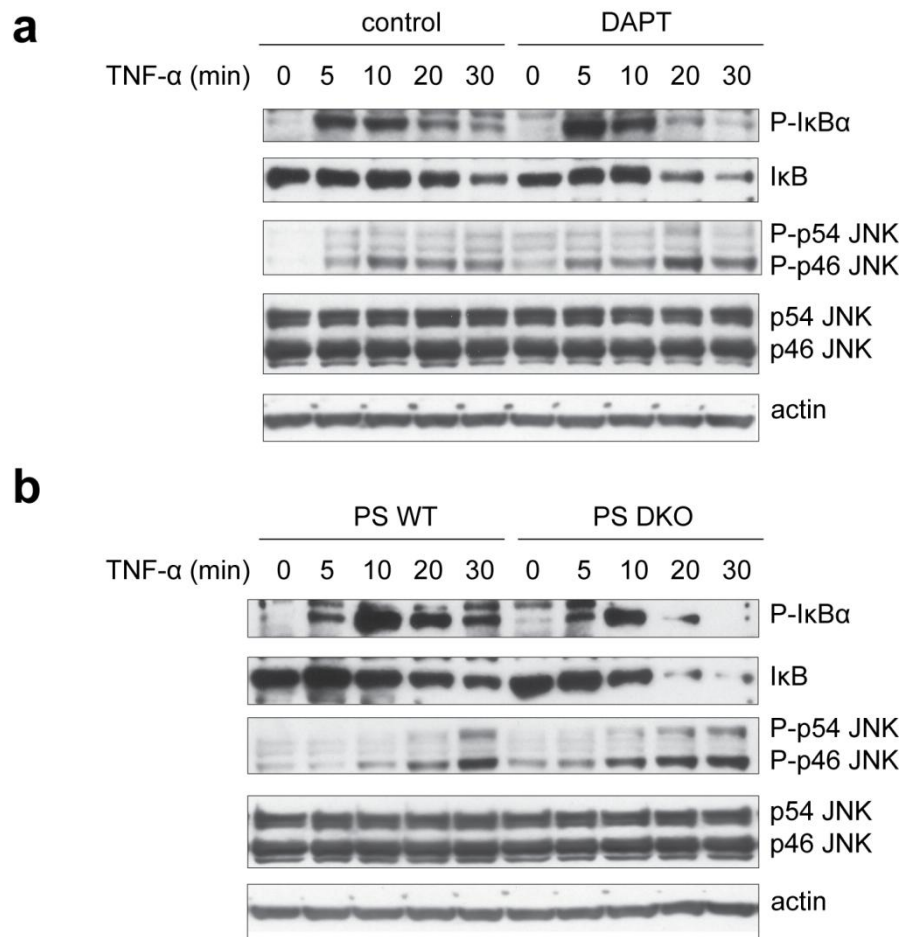


Figure 5.4 Inhibiting γ -secretase-mediated activity does not affect the TNFR1 mediated signalling pathway

(a) MEF from PS wild type and PS double knock-out mice, were serum starved overnight and pre-treated with γ -secretase inhibitor, DAPT (10 μ M; 8h). Following pre-treatment cells were treated with murine TNF α for increasing time as indicated. Cells were lysed and probed for the indicated antibodies. **(b)** MEF from PS wild type mice, were serum starved overnight and treated with murine TNF α for increasing time as indicated. Cells were lysed and probed for the indicated antibodies. The data is representative of three independent experiments.

5.2.5 PS2 affects JNK activation in MEFs in TNFR1 signalling pathway

The above data shows that inhibiting γ -secretase activity does not affect TNF α -mediated signalling pathways, and our previous data shows that TNFR1 undergoes PS1-dependent γ -secretase-mediated cleavage. To explore the possible involvement of PS2 in TNFR1 signalling, we used PS1 single knock-out (PS1KO) and PS2 single knock-out (PS2KO) mouse embryonic fibroblasts (MEFs) to study any defects in TNF α -mediated signalling pathways. PS1KO and PS2KO MEFs were serum starved overnight, following which cells were stimulated with TNF α (10ng/ml), for increasing time. Western blot analysis was carried out to determine the effect of loss of PS1 or PS2 on TNF α -mediated signalling pathway. Immunoblot analysis revealed that while the PSWT and PS1KO MEFs showed comparable TNF α -induced activation of JNK1/2 and NF- κ B (**Figure 5.5 lane 1 to 10**), the loss of PS2 was selectively associated with loss of TNF α -induced JNK1/2 activation. PS1KO MEFs are not defective in either the activation of JNK1/2 or NF- κ B (**Figure 5.5 lane 11 to 15**). Collectively this data shows that TNF α -mediated JNK1/2 activation is dependent upon PS2-mediated γ -secretase independent function.

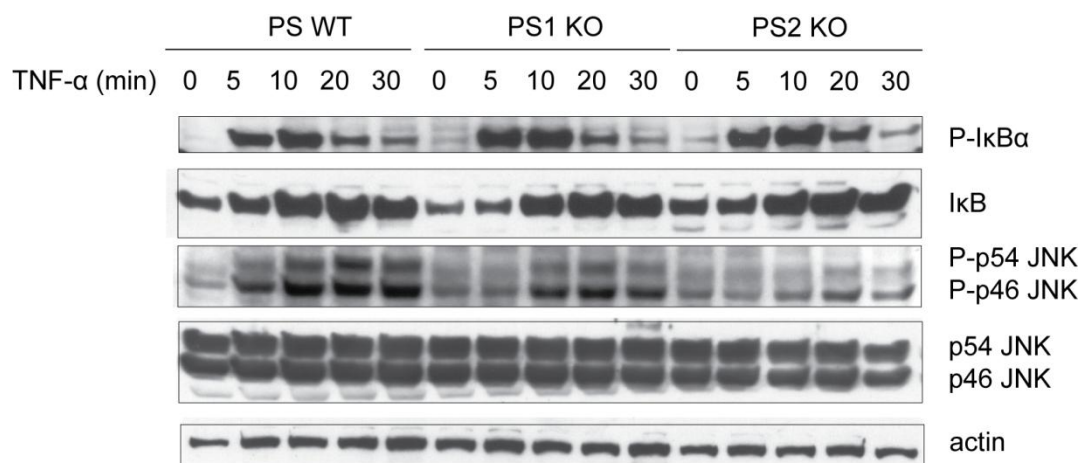


Figure 5.5 PS2 KO MEF cells are defective in activation of JNK1/2

MEF from PS1 and PS2 single knock-out mice, were serum starved overnight and treated with murine TNF α for increasing time as indicated. Cells were lysed and probed with the indicated antibodies. The data is representative of three independent experiments.

5.2.6 Affect of loss of PS on TNF α -induced apoptosis

Following TNF α stimulation, TNFR1 gets internalized and recruits death adaptor proteins to initiate apoptotic signal in the cells, where the activation of JNK has been shown to affect TNF induced apoptosis (Silke, 2011). To examine whether loss of γ -secretase activity is also involved in the apoptosis pathway, we treated PS wild-type cells with TNF α and cycloheximide with or without DAPT (10 μ M/ml; 8h) for indicated times (**Figure 5.7a**). Alternatively, PS wild-type and double knockout cells were also employed in this study (**Figure 5.7b**). Cells were lysed and immunoblotted for cleaved PARP. Analysis revealed that the loss of γ -secretase activity enhanced TNF α -induced PARP cleavage significantly suggesting that pharmacological inhibition of γ -secretase increases susceptibility to apoptosis. However, no significant increase in TNF α -induced PARP cleavage was observed in PS DKO cells.

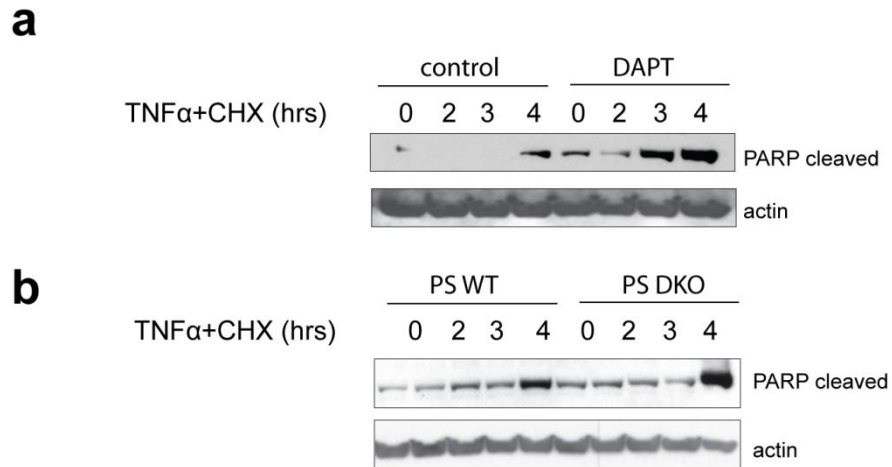
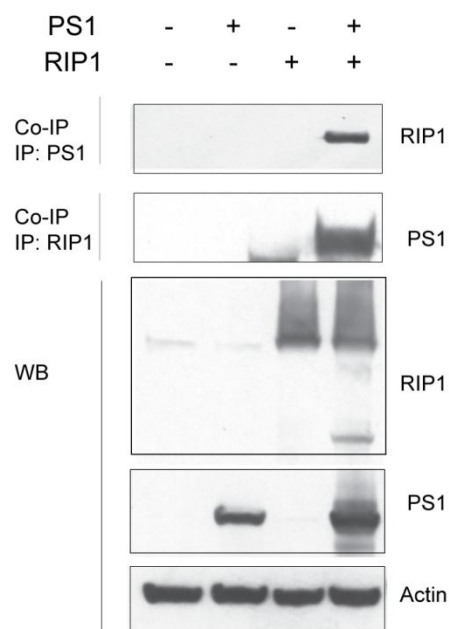
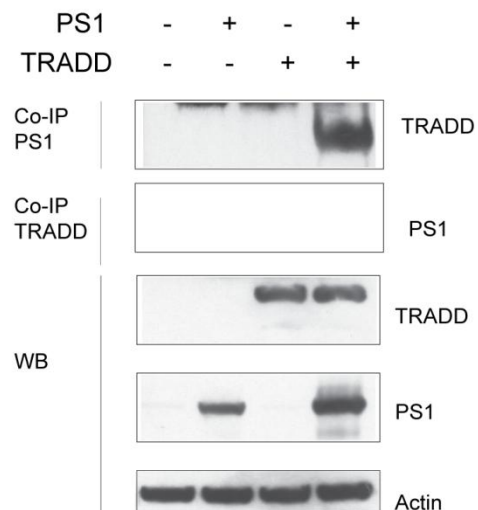


Figure 5.6 Loss of γ -secretase activity increases susceptibility to apoptosis

(a) PS wild-type MEFs were either left untreated or pre-treated with DAPT (10 μ M/ml; 8h), following which they were treated with TNF α and cycloheximide for indicated time points. **(b)** PS wild-type and PS double knockout MEFs were TNF α and cycloheximide for indicated time points. Cells were lysed and immunoblotted using anti-cleaved PARP antibody. The data is representative of two different experiments.

5.2.7 Interaction between PS1 and TNFR1 signalling adaptor proteins

So far our data shows that TNFR1 undergoes PS1-dependent γ -secretase-mediated cleavage and in contrast loss of PS2 and not PS1 affects TNF- α mediated activation of JNK. Interestingly, PS proteins are known to interact with a diverse array of proteins and PS1 and PS2 have been shown to co-regulate each others' expression through p53-dependent mechanisms, which also play an important role in triggering apoptosis (Roperch et al., 1998; Smith et al., 2002b). We have also shown that PS1 and PS2 associate with several adapter proteins (TRAF6, TRAF2, IRAK2) involved in IL-1/TLR and TNF/NGF signalling pathways. In this context, we explored the possibility of an interaction between PS1 and RIP1 which is recruited to TNFR1 in Complex I following TNF α -stimulation. HEK293T cells were transiently transfected with PS1 and co-transfected with RIP1 and thirty hours post-transfection cells were lysed and immunoblotted with anti-PS1, anti-RIP1 and anti- β actin antibody to confirm the expression of constructs and equivalency of loading. After confirming the equivalent expression of the various plasmids, cells were lysed under non-denaturing conditions. PS1 was immunoprecipitated with an anti-PS1 antibody followed by western blotting with anti-RIP antibody to detect RIP1 co-immunoprecipitated with PS1 (**Figure 5.7a**). Simultaneously RIP1 was also immunoprecipitated with anti-RIP1 antibody followed by western blotting with anti-PS1 antibody to detect PS1 co-immunoprecipitated with RIP1 (**Figure 5.7a**). Using a similar experimental set up interaction between PS1 and TRADD was also studied. PS1 was immunoprecipitated with an anti-PS1 antibody followed by western blotting with anti-TRADD antibody to detect TRADD co-immunoprecipitated with PS1 (**Figure 5.7b**). Simultaneously TRADD was also immunoprecipitated with anti-TRADD antibody followed by western blotting with anti-PS1 antibody to detect PS1 co-immunoprecipitated with TRADD. The data revealed that PS1 interacts with RIP1, while PS1 and TRADD interaction was not observed using reverse co-immunoprecipitation approach.

a**b****Figure 5.7 PS1 interacts with RIP1**

HEK293T cells were transiently transfected with the indicated expression constructs. After 30 hours post-transfection cell lysates were immunoprecipitated with the indicated antibodies. Co-immunoprecipitated proteins were detected with an anti-PS1 or anti-RIP1 or anti-TRADD antibodies. Immunoblot analysis of transfected cell lysates with the indicated antibodies confirmed expression of all constructs. The data is representative of three independent experiments.

5.3 Discussion

5.3.1 TRAF2 ubiquitinates TNFR1

In the study presented in chapter 4, we showed that TNFR1 undergoes γ -secretase-mediated proteolysis within the early endosomes. Additionally most known γ -secretase cleavage products have been shown to undergo proteasomal degradation including Notch ICD, Mucin ICD, p75NTR ICD (Julian et al., 2011; Kanning et al., 2003; Urrea et al., 2007). In this context, using lysosomal and proteasomal inhibitors we found that TNFR1 ICD to undergo proteasomal degradation, while in contrast TNFR1 full length receptor and TNFR1 CTF were degraded via a lysosomal pathway (**Figure 5.1a**). Proteasomal degradation has been associated with ubiquitination of targeted proteins. Additionally, Chin and Horwitz linked monoubiquitination of the TNFR1 to its degradation after endocytosis (Chin et al., 2005, 2006). Hence, the importance of monoubiquitination of the TNFR1 in either formation or degradation of the TNFR1 ICD was assessed. Using wild-type Ubiquitin (Ub WT) or the HA-tagged Ubiquitin all lysine mutant (Ub K ϕ) which is defective in forming poly-ubiquitin chains and only undergoes monoubiquitination we propose that polyubiquitination is required for degradation of ICD and monoubiquitination is sufficient for γ -secretase-dependent formation of TNFR1 ICD (**Figure 5.1b**).

TRAF2 is an important adaptor protein in the TNFR1 signalling pathway. Upon TNF α -stimulation TRAF2 is recruited via the amino-terminal domain of adapter protein TRADD, and activates a variety of downstream signalling cascades. Additionally, TRAF2 is the E3 ligase responsible for K63-linked polyubiquitination of RIP1, thus playing an important role in activating the IKK complex (Devin et al., 2000). We have previously demonstrated a role for TRAF6 in ubiquitination and regulated intramembrane proteolysis of IL-1R1 and shown that TRAF2 associates with PS1. In the current study,

we found that TRAF2 lacking its E3 ligase activity shows reduces constitutive and PMA-induced TNFR1 ICD formation, compared to the wild-type TRAF2 expressing cells (**Figure 5.2**). Additionally, TRAF2-induces ubiquitination of TNFR1, thus pointing towards a co-relationship between TRAF2-induced ubiquitination of TNFR1 and its regulated intramembrane proteolysis (**Figure 5.3**).

These results highlight that while polyubiquitination is required for degradation of TNFR1 ICD, TRAF2-mediated polyubiquitination could play a role in formation of TNFR1 ICD. Thus, it is important to study the type of polyubiquitination required for these two process. For instance, it is feasible that K48-linked polyubiquitination is required for the degradation of TNFR1 ICD, while TRAF2-induced K63-linked polyubiquitination is important for its formation. Using Ub mutants with all lysine residues mutated to arginine except residue 48 (Ub K48) or with just the lysine 48 residue mutated to arginine (Ub K48R) the type of polyubiquitination can be studied.

5.3.2 Loss of PS2 affects TNF α -mediated JNK1/2 activation

We showed TNFR1 to undergo PS1-dependent γ -secretase-mediated cleavage, which prompted us to investigate the role of γ -secretase-mediated cleavage in TNF α -mediated signalling pathway. The experiments carried out in PS WT, PS1KO, PS2KO and PSDKO cells revealed no effect of loss of γ -secretase activity on TNF α -induced signalling (**Figure 5.5**). On the contrary, PS2 deficiency was seen to affect TNF α -induced JNK activation. In our attempt to further characterize the γ -secretase-independent role of PS2 on TNF α -mediated signalling, our group carried out studies *in vivo* and detected low level of chemokine CXCL1 in serum of PS2KO animals in response to TNF- α . These observations suggest that PS2 has a novel function in determining TNF- α -mediated activation of JNK and inflammatory responses.

5.3.3 Interaction between PS1 and RIP

Our data shows PS1-dependent γ -secretase-mediated cleavage of TNFR1, while loss of PS2 and not PS1 was found to affect TNF α -mediated signalling pathways. Since, PS1 interacts with TRAF2; we also investigated whether or not PS1 interacts with other TNFR1 signalling adaptor proteins. We used HEK293T cells over-expressing the PS1, RIP1 and TRADD constructs. Using reverse co-immunoprecipitation studies we found PS1 to interact with RIP1, while PS1 did not interact with TRADD in reverse co-immunoprecipitation set up. The use of an over-expression system does limit our study, and experiments need to be carried out in cells endogenously expressing these proteins. Furthermore, TNF α -dependency or regulation of the interaction between these proteins should also be examined. It is feasible that the two PS proteins have distinct roles in TNFR1 signalling pathway. Where, PS1-dependent γ -secretase-mediated cleavage of TNFR1 could function to terminate the signal from complex I, dissociating it and subsequently leading to the formation of complex II. Further experiments studying the interaction of PS proteins and various adaptor proteins, could provide us with a better understanding about the role played by these interactions in mediating cell survival and cell death signals.

Chapter 6

General Discussion

6.1 Overall summary

After their discovery through genetic linkage of families with autosomal dominant forms of FAD, PS proteins have emerged as the catalytic components of γ -secretase protease complexes and are associated with all forms of AD (Lessard et al., 2010; Spasic et al., 2008; Yan et al., 2010). Additionally since the characterization of APP as the first γ -secretase substrate, over 90 new substrates have been identified and characterized, with a wide variety of roles that can be subdivided into functional groups including those involved in cell-cell adhesion, vesicular transport, calcium signalling and apoptosis (Haapasalo et al., 2012). The growing list of γ -secretase substrates has highlighted the biological importance of the PS protein and has added a new perspective to the current concepts regarding the receptor-mediated signalling events.

The conventional view of receptor-mediated signalling holds that the plasma membrane receptors upon binding of their ligands relay signals across membranes following which the activation of intracellular enzymes or changes in second messengers facilitate the propagation of these signals. Alternatively, following ligand binding, most receptors either undergo ectodomain shedding or internalization, thereby negatively regulating or terminating the ligand-receptor generated signal (Hayashida et al., 2010). Once internalized a receptor can be either degraded or recycled back to the plasma membrane. The emergence of regulated intramembrane proteolysis as a distinct mechanism for the regulation of receptor-mediated signalling events now challenges the current concepts regarding receptor-mediated signalling and its regulation, where 1) by removing the membrane bound CTFs responsible for signalling, γ -secretase-mediated cleavage possibly inhibits or initiates key signalling events, 2) processing of proteins by the γ -secretase enzyme complex act to enhance or inhibit its interaction with the cytosolic adaptor proteins, hence directing the output of the signalling event, or 3) the product of the γ -secretase-mediated cleavage in most

cases is a transcriptionally active ICD, which regulates gene transcription (Haapasalo et al., 2012) **Section 1.4.** Additionally, the study of the various PS KO mouse models and transgenic animals has highlighted the role of PS/ γ -secretase in the immune system (Jayadev et al., 2010; Maraver et al., 2007; Tournoy et al., 2004; Yagi et al., 2008; Zhu et al., 2011b). Relevant to the current study it is noteworthy that the IL-1R/TLR and TNF receptor superfamily play important roles in innate immunity, inflammatory and growth-factor responses **Section 1.6.** IL-1R1 and TLR4 are type-1 transmembrane proteins and have no intrinsic enzymatic activity thus they need to recruit multimeric complexes with accessory proteins like IL-1RAcP for IL-1R1 or MD2 and CD14 for TLR4 and cytosolic adapter molecules such as the TRAFs, IRAKs and RIP1 in order to initiate cellular responses (Verstrepen et al., 2008). What is unique about these three receptors is that they all undergo spatially segregated, ligand-induced signalling events initiating from both the cell surface and the endosomes (Brissoni et al., 2006; Kagan et al., 2008; Li et al., 2006; Schneider-Brachert et al., 2004). The aim of this work was to characterize TNFR1 and TLR4 as potential substrates for regulated intramembrane proteolysis and determine the subcellular occurrence of these cleavage events. Further to this, we also studied whether these receptors undergo PS1- or PS2- dependent γ -secretase-mediated cleavage.

Our results point towards a new mechanism for the induction and regulation of signalling events mediated by these receptors. In the current study, in addition to previously described IL-1R1 (Elzinga et al., 2009), we identified TNFR1 and TLR4 as novel γ -secretase substrates, where ectodomain shedding of these receptors was prerequisite for further cleavage by γ -secretase enzyme complex. Along with TNFR1 ectodomain shedding, IL-1R1 and TLR4 were found to undergo constitutive and ligand/PMA-induced secretion of the soluble ectodomain in the extracellular milieu. Similar to CD74 (Becker-Herman et al., 2005), ectodomain shedding of IL-1R1 occurs in the endosome, and the cell subsequently secretes the sIL-1R1 in the extracellular

milieu. This is of particular interest since most released ectodomains in addition to regulating the normal turnover of the receptor also retain their biological activities and compete for binding to receptor ligands. It is also feasible that before being secreted sIL1-R1 directly mediates intracellular signalling events. A study carried out by Heguy and co-workers has shown that in addition to full length ligand bound IL-1R1, ligand bound C-terminal domain truncated IL-1R1 migrates to the nucleus (Heguy et al., 1991). In this context, our finding that IL-1R1 ICD undergoes nuclear translocation, which is dependent upon the NLS sequence 4, is indeed very interesting, and points towards a new mechanism of regulation of IL-1 β -induced signalling. Owing to the highly homologous C-terminus domains of IL-1R1 and TLR4 it will be interesting to investigate whether or not TLR4 ICD also translocates to the nucleus.

These observations are encouraging and supportive of future studies to determine the role played by trafficking and spatially segregated cleavage events in mediating various signalling events, and in turn the role played by PS proteins dependent or independent of their γ -secretase activity in mediating these trafficking events. Based on our results, on completion of our studies we propose the models in **Figure 6.1** and **Figure 6.2**. On basis of data available from literature and results obtained in the current study, it is important to determine the role played by metalloproteinases and γ -secretase-mediated processing of these receptors. Increased levels of both, soluble ectodomains and ligands for these receptors have been observed in several diseases including previously discussed AD.

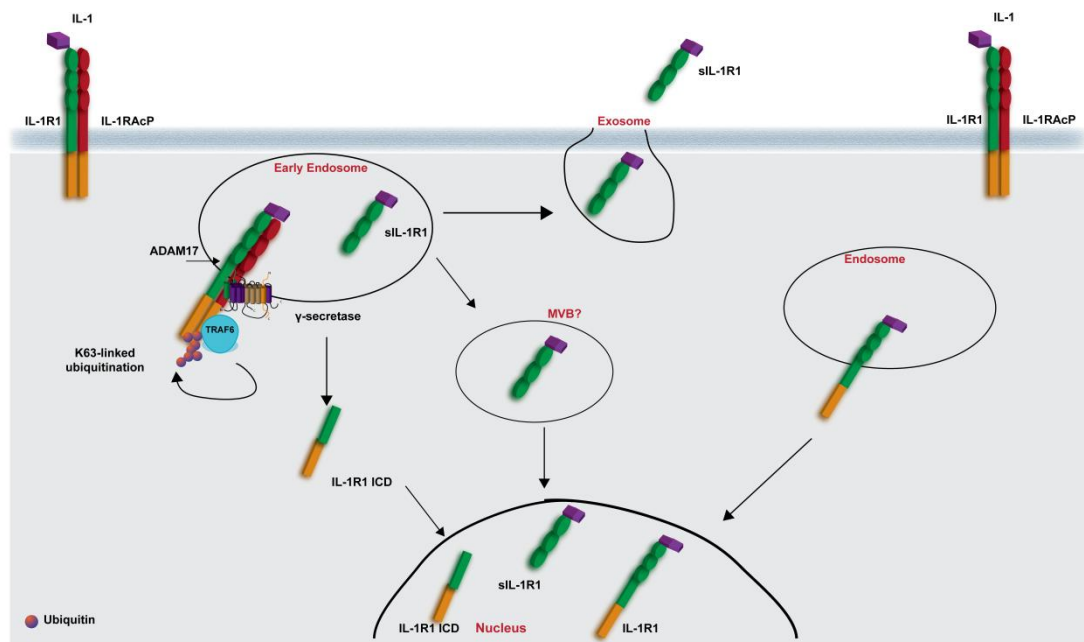


Figure 6.1 Proposed model for the spatial regulation of IL-1R1 signalling and occurrence of ectodomain shedding and subsequent γ -secretase cleavage.

IL-1 stimulation leads to the recruitment of IL-1RAcP forming the IL-1/IL-1R1/IL-1RAcP complex. This complex undergoes clathrin-mediated internalization and is either translocated to the nucleus or traffics into the endosomes, where it undergoes cleavage in the extracellular domain by metalloproteinase, ADAM17, generating a soluble IL-1R1 which is then secreted into the media and has also been reported to undergo nuclear translocation. Ectodomain shedding of IL-1R1 is a pre-requisite cleavage event for subsequent PS1 dependent γ -secretase mediated cleavage. The IL-1R1 ICD thus formed undergoes either proteasomal degradation or translocates to the nucleus. During this proteolytic processing pathway, IL-1R1 also recruits TRAF6 on the endosomes, where it undergoes K63-linked polyubiquitination which in turn enhances the regulated intramembrane proteolysis of IL-1R1 by ADAM17 and subsequently by γ -secretase protease.

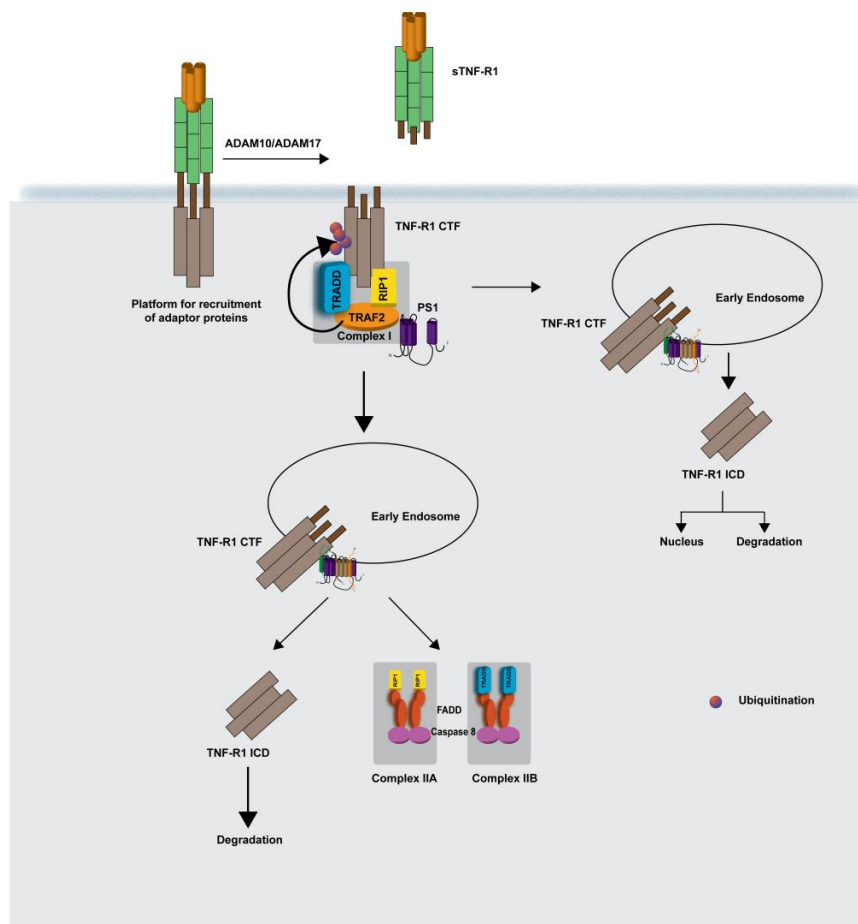


Figure 6.2 Proposed model for TNFR1 and subcellular localization of receptor ectodomain shedding and γ -secretase-mediated cleavage

TNF α -stimulation leads to trimerization of the TNFR1, which is then cleaved in its extracellular domain by ADAMs (ADAM10/17) to generate sTNFR1, secreted in the extracellular milieu. Simultaneously TRADD, RIP and TRAF2 are recruited to TNFR1 on the cell surface, forming Complex I, which signals for cell survival pathways. We propose that PS1 at this stage interacts with RIP1 and TRAF2-possibly as a part of the γ -secretase enzyme complex. This TNFR1 bound Complex I undergoes clathrin-mediated internalization followed by γ -secretase-mediated cleavage. Based on preliminary data we propose that γ -secretase-mediated cleavage of TNFR1 regulates the spatial segregation of TNFR1-mediated signalling pathways.

6.2 Future Perspectives

6.2.1. Characterizing the role played by TRAF-induced ubiquitination of IL-1R1 and TNFR1

TRAFs act as the major signal transducers of TNFR and the IL-1/TLR superfamily and their role in both adaptive and innate immunity has also been highlighted (Y. Wang et al., 2010; Xie et al., 2008). In the current study we demonstrated TRAF6-induced ubiquitination and cleavage of IL-1R1. Furthermore, it has been shown that inhibition of IL-1R1 endocytosis prevents its interaction with TRAF6 (Q. Li et al., 2006). Thus we hypothesized that IL-1R1 would be ubiquitinated by TRAF6 either in the endosome or post-endosomal compartments. To investigate this theory, IL-1R1 was co-transfected with TRAF6 and dominant negative dynamin construct, which is defective in clathrin-mediated internalization. The effect of dynamin dominant negative construct was observed on the TRAF6-induced regulated intramembrane proteolysis and ubiquitination of IL-1R1 and was found to inhibit internalization of IL-1R1 to endosome preventing both TRAF6-induced regulated intramembrane proteolysis and ubiquitination of IL-1R1. Further to this, other experiments carried out in our group using dominant-negative Rab constructs such as Rab4 required for trafficking of protein through fast recycling pathway, Rab11 for the slow recycling pathway and Rab7 for trafficking to the lysosome showed an increase in TRAF6-induced cleavage and ubiquitination of IL-1R1. Thus, demonstrating that TRAF6-mediated ubiquitination of IL-1R1 occurs in the endosomes. Further to this we also observed that TNFR1 undergoes TRAF2-induced ubiquitination.

An important factor to consider here is that ubiquitination of target proteins is a regulatory event in receptor endocytosis and endosomal trafficking (Mukhopadhyay et al., 2007). In this regard, the finding that the subcellular trafficking of IL-1R1 and TNFR1 mediates their ectodomain shedding and γ -secretase-mediated cleavage points

towards a role of TRAFs in mediating trafficking of IL-1R1 and TNFR1. Thus there is a need to characterize these interactions, and determine how γ -secretase-mediated cleavage of these receptors is regulated by TRAF-mediated ubiquitination which could further provide an insight into the mechanism behind the regulation of receptor-mediated signaling pathways. This could be carried out by studying the cell surface localization of IL-1R1, and its internalization and trafficking in wild-type TRAF6 and TRAF6 knockout cells. This could also be carried out in PSKO, PS1KO and PS2KO cells to further determine role of PS proteins independent of their γ -secretase activity. Additionally, IL-1R1 signaling is spatially segregated and majorly mediated at the endosomal compartments. It is thus, feasible that TRAF6-mediated ubiquitination of IL-1R1 functions to positively recruit the signalling adaptor molecules and formation of IL-1R1 signalling complexes. This is supported by the observation that, TRAF6-induced ubiquitination of IL-17 receptor is important for IL-17 associated signaling (Hartupée et al., 2009). Therefore, studying the interactions between IL-1R1 and its adaptor molecules, and even PS proteins and members of the IL-1R1 signalling pathway could provide an insight into the role played by TRAF6 in IL-1R1 signalling pathway.

Similar to TRAF6-induced ubiquitination of IL-1R1, we observed TRAF2-induced ubiquitination of TNFR1 which was diminished in the presence of dominant negative TRAF2 lacking E3 ligase activity. TRAF2 was originally identified by its association with TNFR2 (Rothe et al., 1994), and TRAF2 has been reported to play an important role in anti-apoptotic signalling, JNK activation and perinatal survival (Devin et al., 2000; S. Y. Lee et al., 1997; Yeh et al., 1997). As the next step it is important to determine the specificity of TRAF2-induced ubiquitination of TNFR1 to assure that it is unique to TRAF2 and whether the effect is redundant among TRAF family members. Due to low expression levels of endogenous TNFR1 cleavage fragments, the experiments to study the effect of TRAF2 on ubiquitination of TNFR1 require to be carried out in an over-expressed cell system. Hence, it is important to include an E3 ligase outside of TRAF

family to validate that the effect seen on co-expression of TRAF2 is specific. In this regard, Parkin, an E3 ubiquitin ligase which possesses two RING finger domains and can mediate monoubiquitination, and both K48- and K63-linked polyubiquitination could be used. Similar to IL-1R1, we also checked whether the lack of TRAF2 E3 ligase activity had any effect on TNFR1 ICD formation. Due to reduced expression of TNFR1 in cells co-transfected with TRAF2 DN our result was not conclusive. Hence, it is important to carry out similar experiments in wild-type TRAF2 and TRAF2 knockout cells. ELISA for sTNFR1 in these cells would further validate whether TRAF2 is required for ectodomain shedding and just affects γ -secretase-mediated cleavage of TNFR1.

6.2.2 Nuclear localization of IL-1R1 ICD

Cleavage of IL-1R1 by regulated intramembrane proteolysis is important for several reasons. Firstly, the cleavage in the extracellular domain by ADAM17, leads to the secretion of sIL-1R1 in the extracellular milieu. sIL-1R1 has been previously suggested to regulate IL-1R1-mediated signalling pathways by sequestering IL-1 β , or terminating the signal from IL-1R1. However, the observation that IL-1R1 undergoes ectodomain shedding in the endosome challenges the current concept and indicates that sIL-1R1 could play a more important role than just regulating IL-1R1-mediated signalling pathways. Secondly, defective signalling in cells expressing C-terminal truncated IL-1R1 has been shown (Heguy et al., 1991). Thus highlighting the importance of γ -secretase cleavage generated IL-1R1 ICD. This could be attributed to the interaction between the IL-1R1 ICD and various adaptor molecules involved in mediating the IL-1 β induced signalling. In this context, in addition to previously reported migration of ligand-bound IL-1R1 to the nucleus, in the current study we demonstrated that IL-1R1 ICD translocates to the nucleus. The IL-1R1 ICD has four NLS sequences, and mutating the lysine 548 residue to arginine in NLS sequence 4 inhibits its translocation to the nucleus.

To further study the role of IL-1R1 ICD and its translocation to the nucleus, it is important to map the γ -secretase cleavage site. In an attempt to map the cleavage site our group employed the approach as used by Lichtenthaler and group to map APLP2 cleavage site by ADAM10, BACE1 and γ -secretase enzyme complex (Hogf et al., 2011). N- and C-terminus tagged IL-1R1 constructs was generated, over-expressed in a cell system. On ligand/PMA stimulation the IL-1R1 ICD generated was immunoprecipitated using the antibody against the C-terminus tag. However, due to low expression levels of IL-1R1 ICD this technique was not successful. Alternatively, a site directed mutagenesis approach mutating alternate residues within the transmembrane domain of IL-1R1 could be used. Once the site is mapped, it could be used to generate a non-cleavable, constitutively cleaved and IL-1R1 ICD constructs. Using these constructs and monitoring the effect of these constructs on IL-1 β induced signalling the role played by the translocation of IL-1R1 ICD to the nucleus can be more extensively studied. IL-1R1 ICD construct could be further employed to generate IL-1R1 ICD specific antibody to perform chromatin immunoprecipitation (ChIP) and detect its association with the promoter of the several genes. Additionally microarray analysis could be used in order to identify DNA binding sites of the IL-1R1-ICD.

In addition to IL-1R1 ICD reported here, over time ICDs for several γ -secretase substrates have been shown to migrate to the nucleus and regulate gene transcription (**Table 1.2**). The cytosolic domain of IL-1R1 and TLR4 are highly homologous, an alignment of TLR family members and their intracellular cytosolic domain with IL-1R1 is depicted in **Figure 6.3**. The alignment results indicate conservation amongst the residues of NLS sequence 1 and 4 of IL-1R1 ICD with TLR family members. In particular TLR3 and TLR4 show consensus within the NLS sequence 1, though not highly conserved, it would be interesting to study whether TLR4 ICD translocates to the nucleus on ligand stimulation.

NLS conservation - IL1R and TLR

				NLS1																											
IL1R 1	TM-ICD	(98)		G	E	D	I	V	E	V	I	N	E	N	V	K	K	-	S	R	R	L	I	I	I	L	V	R	E	T	S
TLR 3	TM-ICD	(92)		G	V	F	E	L	E	A	I	V	N	S	I	K	R	-	S	R	K	I	I	F	V	I	T	H	H	L	L
TLR 1	TM-ICD	(97)		G	K	S	I	V	E	N	I	I	T	C	I	E	K	-	S	Y	K	S	I	F	V	L	S	P	N	F	V
TLR 6	TM-ICD	(97)		G	K	S	I	V	E	N	I	I	N	C	I	E	K	-	S	Y	K	S	I	F	V	L	S	P	N	F	V
TLR 10	TM-ICD	(99)		G	K	S	I	S	E	N	I	V	S	F	I	E	K	-	S	Y	K	S	I	F	V	L	S	P	N	F	V
TLR 2	TM-ICD	(94)		G	K	W	I	I	D	N	I	I	D	S	I	E	K	-	S	H	K	T	V	F	V	L	S	E	N	F	V
TLR 4	TM-ICD	(84)		G	V	A	I	A	N	I	I	H	E	G	F	H	K	-	S	R	K	V	I	V	V	S	Q	H	F	I	
TLR 7	TM-ICD	(97)		G	Q	P	V	I	E	N	I	S	Q	S	I	Q	L	-	S	K	K	T	V	F	V	M	T	D	K	Y	A
TLR 8	TM-ICD	(98)		G	L	A	I	I	D	N	I	M	Q	S	I	N	Q	-	S	K	K	T	V	F	V	L	T	K	K	Y	A
TLR 9	TM-ICD	(98)		G	K	T	I	F	E	N	I	W	A	S	V	Y	G	-	S	R	K	T	I	F	V	L	A	H	T	D	R
TLR 5	TM-ICD	(98)		G	E	N	R	I	A	N	I	Q	D	A	I	W	N	-	S	R	K	I	V	C	L	V	S	R	H	F	L
Consensus				(111)	G		I	I	E	N	I	I	S	I		K			S	R	K	T	I	F	V	L	S		F	V	

				NLS4										NLS3										NLS2													
IL1R 1	TM-ICD	(188)		P	Q	S	A	K	T	R	E	F	W	K	N	V	R	Y	H	M	P	V	Q	R	R	S	P	S	S	K	H	Q	L	L	S	P	A
TLR 3	TM-ICD	(182)		---	I	G	A	F	R	H	K	L	Q	V	A	I	G	S	K	N	S	V	H	---	---	---	---	---	---	---	---	---	---	---	---	---	---
TLR 1	TM-ICD	(186)		---	R	G	L	F	W	A	N	L	R	A	A	I	N	I	K	L	T	E	Q	A	K	---	---	---	---	---	---	---	---	---	---	---	---
TLR 6	TM-ICD	(186)		---	R	G	L	F	W	A	N	L	R	A	A	I	F	N	M	K	L	T	L	V	T	E	N	N	D	V	K	S	---	---	---	---	
TLR 10	TM-ICD	(188)		---	C	G	L	F	W	A	N	L	R	A	A	I	N	V	N	V	L	A	T	R	E	M	Y	E	L	Q	T	F	T	---	---	---	
TLR 2	TM-ICD	(183)		---	R	E	G	F	W	V	N	L	R	A	A	I	K	S	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
TLR 4	TM-ICD	(173)		---	R	H	I	F	W	R	R	L	R	K	A	L	L	D	G	K	S	W	N	P	E	G	T	V	G	T	G	C	N	---	---	---	
TLR 7	TM-ICD	(183)		---	H	P	Y	F	W	Q	C	L	K	N	A	L	A	T	D	N	H	V	A	Y	S	Q	V	F	K	E	T	V	---	---	---	---	
TLR 8	TM-ICD	(184)		---	E	G	L	F	W	Q	T	L	R	N	V	L	T	E	N	S	R	Y	N	N	M	Y	V	D	S	I	K	---	---	---	---		
TLR 9	TM-ICD	(184)		---	Q	R	S	E	F	W	A	Q	L	G	M	A	L	T	R	D	N	H	H	F	Y	N	R	N	F	C	Q	G	P	T	---	---	
TLR 5	TM-ICD	(186)		---	V	G	W	F	L	H	K	L	S	Q	Q	L	L	K	E	K	E	K	K	D	N	N	I	P	L	Q	T	---	---	---	---	---	
Consensus				(204)		G		F	W		N	L	R		A	I																					

				NLS1																		
IL1R 1	TM-ICD	(104)		V	I	N	E	N	V	K	K	S	R	R	L	I	I	I	L	V	R	E
TLR 3	TM-ICD	(98)		A	I	V	N	S	I	K	R	S	R	-	K	I	I	F	V	I	T	H
TLR 4	TM-ICD	(90)		N	I	I	H	E	G	F	H	K	S	R	K	V	I	V	V	S	Q	
Consensus				(108)		I	I		I	K	K	S	R	R	K	I	I	V	V	S		

Figure 6.3 Alignment of cytosolic domain of TLR family members and IL-1R1 ICD

Sequence alignment of IL-1R1 ICD and the putative ICD of several TLR family members, showing highly conserved NLS sequence 1 amongst IL-1R1 and TLR4.

6.2.3 PS proteins and their interactions with the IL-1R1, TLR4 and TNFR1 adaptor molecules

We identified PS2 as an important determinant of TLR4, IL-1R1 and TNFR1 signalling pathways thus, demonstrating a role of PS2 in innate immune responses. However, we did not see similar effects in cell system deficient in both the PS proteins. This is partly supported by the observation that PS1^{-/+} PS2^{-/+} mice remain healthy indicating a critical border below which the drop of PS gene dosage and function faces serious risks (Tournoy et al., 2004). However, it has been not been determined whether this γ -secretase independent signalling deficit is due to loss of PS2 interaction with adaptor molecules involved in the signalling pathways, or due to an indirect effect of loss of PS2 expression, for instance wild-type and FAD-linked PS2 down-regulates PS1 expression through p53-dependent mechanism (H. O. Smith et al., 2002), and thus it is feasible that loss of PS2 expression in our cell system enhances the PS1 protein expression and PS1 gain of function. Hence, the phenotype observed in cell/animal models lacking PS2 could be a result of PS1 gain in function.

Previous studies by our group have been focused on the PS1 protein and its interaction with various adaptor molecules involved in the IL-1R1 and TLR4 signalling pathways. We have shown that PS1 interacts with TRAF6 and IRAK1 (Elzinga et al., 2009). Preliminary experiments also revealed that PS proteins do not interact with either Mal or p62 and therefore have preference for interaction with some of the signalling components over others. In the current study, we further showed an interaction between PS1 and RIP1, an adaptor molecule involved in TNFR1-mediated signalling pathway. Additionally it would be of interest to determine whether binding of PS1 to RIP1 is either direct or mediated by another protein and whether this interaction is a ligand dependent event. To further characterize the role played by the interaction between PS1 and RIP1, PS1 mutants or competitive binding peptides could be used to reveal the contribution of PS1-RIP1 binding to TNF α -mediated signalling pathway.

Reciprocally, the sites on RIP1 at which PS1 interacts should be mapped, mutated and used to investigate the implications of RIP1 binding to PS1. It has become evident that different adaptor proteins are temporarily recruited to the IL-1R1, TNFR1 and TLR4 signalling pathways; furthermore the spatial segregation of the signalling pathways activated has been well characterized. Thus, it is important to further study the subcellular compartments at which these interactions occur. Our preliminary data suggests that preventing γ -secretase-mediated cleavage of TNFR1 using DAPT, a γ -secretase inhibitor decreases the recruitment of RIP1, while enhancing TRADD interaction with TNFR1 (**Figure 6.4b**). We propose that this could play a role in TNFR1-mediated apoptotic signalling pathways (**Figure 6.4a**). However, it still remains to be determined whether PS1 interacts with RIP1 independently or as a part of γ -secretase enzyme complex. Therefore immunoprecipitation experiments to study the interactions between RIP1 and other components of γ -secretase enzyme complex such as NCT, Aph-1 or Pen-2 need to be carried out.

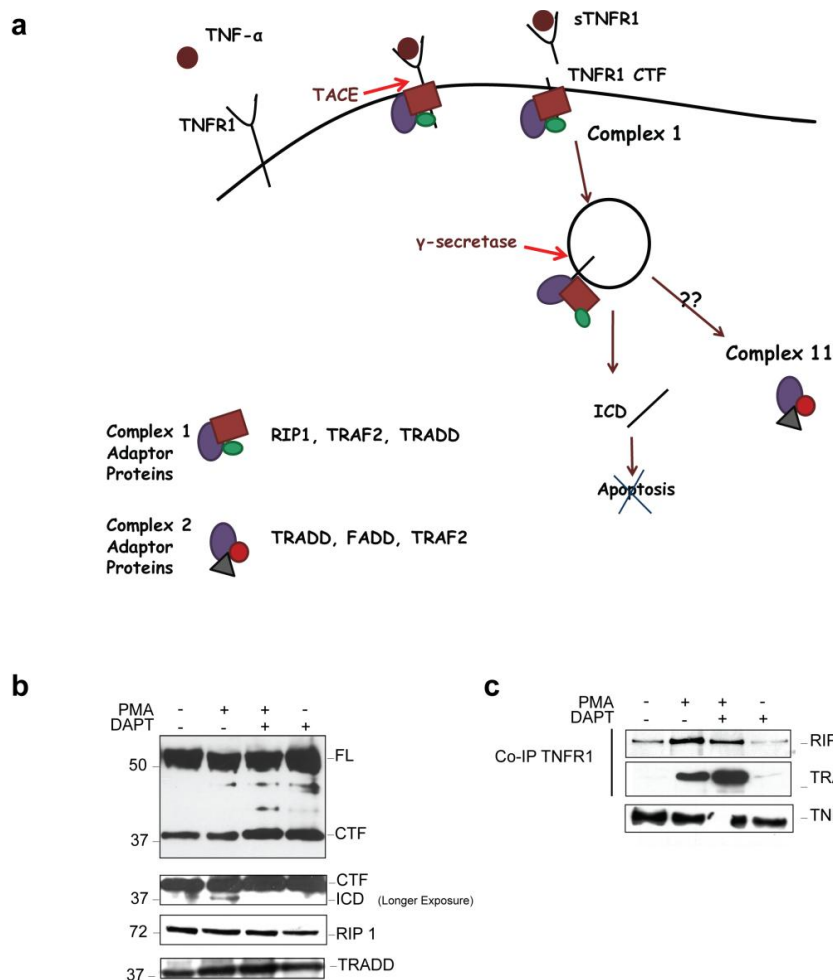


Figure 6.4 Spatial segregation and recruitment of RIP1/TRADD to TNFR1 complex I

(a) Proposed model controlling the spatial segregation of TNFR1-mediated signalling by complex I and complex II. **(b)** HEK293T cells were transfected with TNFR1 and co-transfected with RIP1 and TRADD. Thirty hours post-transfection cells were pre-treated with DAPT (10 μ M/ml; 8h) followed by treatment with PMA (200ng/ml). Cells were lysed and immunoblotted using C-terminus specific anti-TNFR1, anti-RIP1, anti-TRADD and anti-actin antibody. **(c)** Cells were lysed under non-denaturing conditions and co-immunoprecipitated using C-terminus specific anti- TNFR1 antibody. Following which they were probed with either anti-RIP1 or anti-TRADD antibody. The data is representative of two experiments.

6.2.4. Determining the role of TNF-R1, IL-1R1- and TLR4-ICD

Using a series of γ -secretase inhibitors and PS deficient cells and mice, work in our laboratory showed that IL-1 β -, TNF- α and LPS-mediated signalling was independent of γ -secretase activity. On the contrary, data presented in the current research work is indicative of the importance of PTM and spatial regulation of these γ -secretase-mediated cleavage events, which is further highlighted by the translocation of IL-1R1 ICD to nucleus and presence of NLS sequences within the TLR4 ICD. PS proteins have been implicated in several other processes independent of their γ -secretase-mediated activity. Thus, it is important to further investigate the role of these cleaved fragments. Cleavage-resistant chimeric IL-1R1, and TNFR1 similar to the TLR4FasTM construct (**Figure 3.20**) will be an important tool to test the role of TNF-R1, IL-1R1 and TLR4 proteolysis in immune responses and examine whether or not proteolysis of TNF-R1, IL-1R1 and TLR4 and generation of receptor-derived ICDs enhance TNF- α , IL-1 β - or LPS signalling or acts as a dominant inhibitor of cell responsiveness. The activation status of MAPK, NF- κ B and IRF-3 by western blot analysis, luciferase reporter assays and cytokine production could be assessed. Furthermore, it will also be important to determine whether or not the cleavage fragments of these receptors show enhanced processing or accumulate in certain disease conditions such as in AD patients or autoimmune disorders like autoimmune lymphoproliferative syndrome (ALPS) and SLE. To date, blocking IL-1 signaling is the most effective therapy in many auto-inflammatory disorders such as familial cold autoinflammatory syndrome, Muckle-Wells syndrome, chronic infantile neurologic cutaneous and articular syndrome, and TNF-receptor associated periodic syndrome. Thus, it will be important to study role played by the processing of these receptors and their importance in autoimmune and auto-inflammatory diseases.

6.3 Conclusion

Since the discovery of AD, various theories have been proposed for the onset of this disease ranging from 1) exacerbation of aging, 2) degeneration of anatomical pathways, including the cholinergic and cortico-cortical pathways, 3) an environmental factor such as exposure to aluminium, head injury, or malnutrition, 4) genetic factors including mutations of *APP* and *PSEN* genes and allelic variation in *Apo E*, 5) mitochondrial dysfunction, 6) a compromised blood brain barrier, (7) immune system dysfunction, and (8) infectious agents (Armstrong, 2013). What remains common in the background is the γ -secretase enzyme complex which regulates the processing and turnover of a range of proteins which are implicated in these pathways. For instance, altered γ -secretase activity and specificity with aging is considered as a possible mechanism for AD (Placanica et al., 2009). The cholinergic hypothesis of AD states that changes in expression of NGF, its precursor proNGF, the high and low NGF receptors, trkA and p75NTR, respectively which are γ -secretase substrates, may all contribute to the cholinergic dysfunction similarly, altered APP processing by γ -secretase contributes to the amyloid cascade hypothesis (Hardy et al., 1992; Schliebs et al., 2011). All these observations not only highlight the importance of γ -secretase enzyme in AD, but point out a generic role of this enzyme. The genetic factors contributing to AD include mutations in *PSEN* genes, which are the catalytic components of γ -secretase, and mutations within *PSEN* alter its activity. Thus, can it be said that AD is a result of altered γ -secretase enzyme activity which can be attributed to environmental factors, aging or genetic predisposition due to mutations within its catalytic components resulting in altered processing of its substrates like APP, p75NTR, IL-1R1, IL-1R2 contributing to AD symptoms?

In the current research study we focused on innate immune receptors and in addition to previously determined IL-1R1, characterized TLR4 and TNFR1 as novel γ -secretase substrates. Considering our first hypothesis that AD is attributed to altered γ -secretase

enzyme activity, there was plenty of evidence to link these three receptors and γ -secretase enzyme complex. Increased levels of sIL-1R2 (IL-1R2 is a γ -secretase substrate and IL-1R1 decoy receptor) in cerebrospinal fluid of AD patients, increased IL-1 β expression by glial cells associated with amyloid plaques, increased levels of IL-1 β in blood and plasma of AD patients; increased TNF α in cerebrospinal fluids, upregulation of TNFR1 expression in AD brain, and deletion of TNFR1 resulting in decreased amyloid plaques formation and inflammation; upregulation of *TLR4* mRNA in APP transgenic mice and increased TLR4 in AD brain, with further evidence of defective TLR4-mediated signalling in PS-deficient B cells **Section 1.6**. Based on this evidence it was not surprising to find out that along with previously identified 91 γ -secretase substrates, these receptors were also in the list. However, further characterization of these receptors led to a few very interesting findings and challenged the current concepts regarding receptor-mediated signalling events and immune signalling.

Firstly, in addition to previously reported TNFR1, we found IL-1R1 and TLR4 to undergo ectodomain shedding. Though initially the ectodomain shedding was regarded as a means of terminating ligand initiated signalling, in past few years this concept has been challenged, where the soluble ectodomains still retain their biological activity. Furthermore, the finding that IL-1R1 undergoes this cleavage in the endosome coupled with the demonstration that ligand bound C-terminal truncated IL-1R1 translocates to the nucleus points towards a role for soluble receptor ectodomains beyond the termination of signals. Further studies are needed to better understand the roles played by these soluble ectodomains. Another important concept that emerges from our findings is a probable role of γ -secretase cleavage in mediating the spatial segregation of receptor complexes. These three receptors undergo spatially segregated complex formations and the signals initiated. Thus the finding that the subcellular location for ectodomain shedding and γ -secretase-mediated cleavage of these substrates varies points towards a role of γ -cleavage beyond receptor turnover and terminating the signals mediated by its substrates. This is

further supported by translocation of IL-1R1 ICD to nucleus and presence of NLS sequence within the TLR4 ICD.

When our group investigated the role of γ -secretase cleavage on signalling events mediated by IL-1R1, TLR4 and TNFR1, to our surprise they were independent of γ -secretase activity but dependent upon PS2, supporting previous findings suggesting roles of PS proteins independent γ -secretase (**Reviewed in Section 1.3**) and highlighting molecular and functional relationships between PS proteins and innate immune signalling pathways. However, this does not refute the importance of ectodomain shedding and γ -secretase-mediated cleavage of these receptors but points towards a distinct biological function. More work is needed to further study the importance of regulated intramembrane proteolysis independent of loss of PS2 function. In this context the preliminary data pointing towards the spatial segregation of TNFR1 complex I and complex II and its regulation by γ -secretase complex could be a starting point.

Appendix

Appendix Table 1 Reagents used in the research study

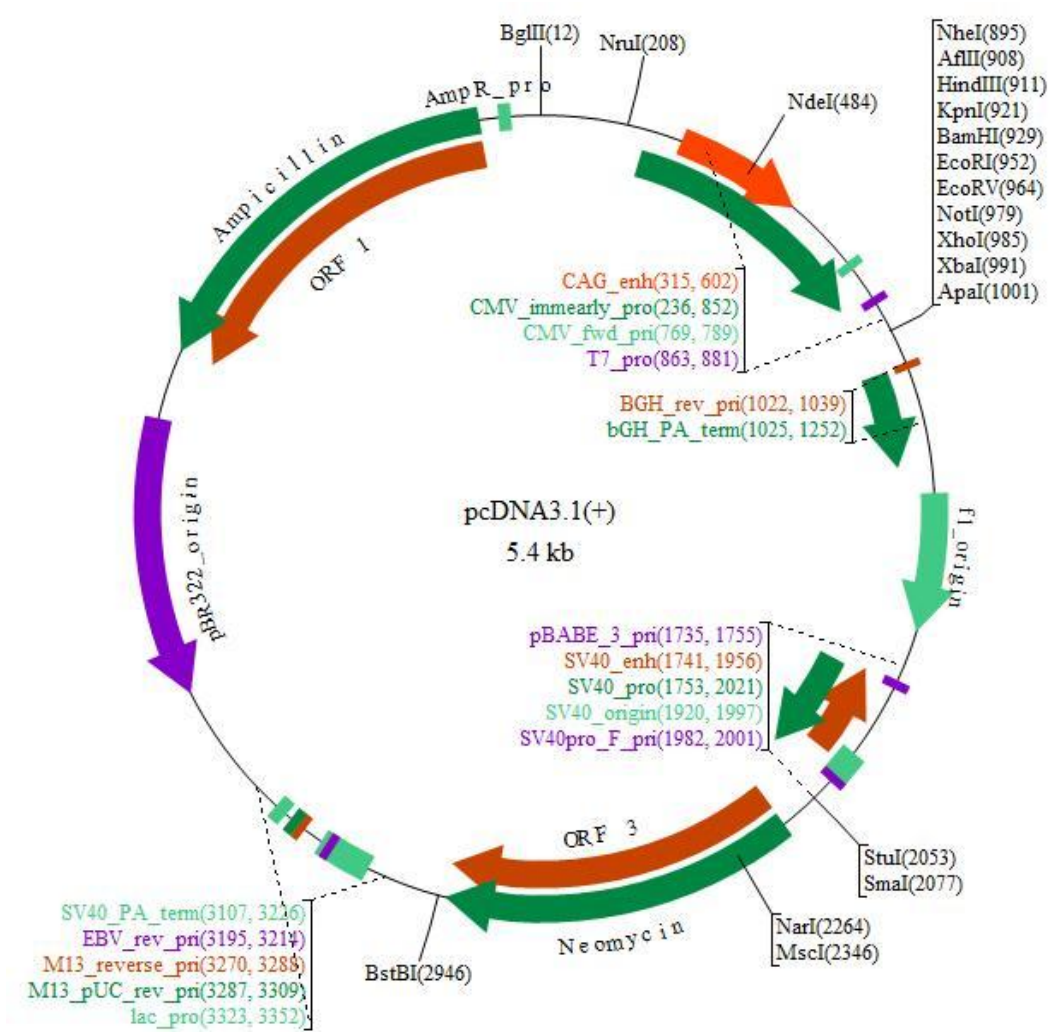
Reagent	Catalogue No.	Company
Bicinchonnic Acid	23223/23224	Pierce Biotechnology
Chemiluminiscent detection kit (ECL)	RPN2106	Amersham Pharmacia Biotech
4',6'-diamidino-2-phenylindole DAPI	D1306	Invitrogen
DNA ladder	N3232S	NEB Ladder
DNA midiprep kit	A2495	Promega-Millipore
γ -secretase inhibitors-DAPT	565770	Calbiochem
High fidelity Pfu polymerase	600382	Stratgene
KOD Hot start DNA polymerase	71086	Calbiochem
Ligands Murine Interleukin-1 β Human Interleukin-1 β Murine Tumor necrosis factor- α Human Tumor necrosis factor- α Human Lipopolysaccharide	211-11B SRP3083 315-01A 654205 437620	Peprotech Sigma Peprotech Calbiochem Calbiochem
Nitrocellulose membrane		Schleicher and Schull
Phorbol 12-myristate 13-acetate (PMA)	524400	Calbiochem
Prestained molecular weight protein marker	161-0374	Bio-Rad
Prolong gold antifade mounting media	P36930	Invitrogen
Protease inhibitor cocktail tablets		Roche
	101241	Invitrogen
Restriction Enzymes		New England Biolabs
T4 DNA ligase	M0202S	New England Biolabs
Turbofect transfection medium	R0531	Promega-Millipore
Western blotting filter paper		Schleicher and Schull

Appendix Table 2 Plasmids employed in the study, their tags and sources procured from.

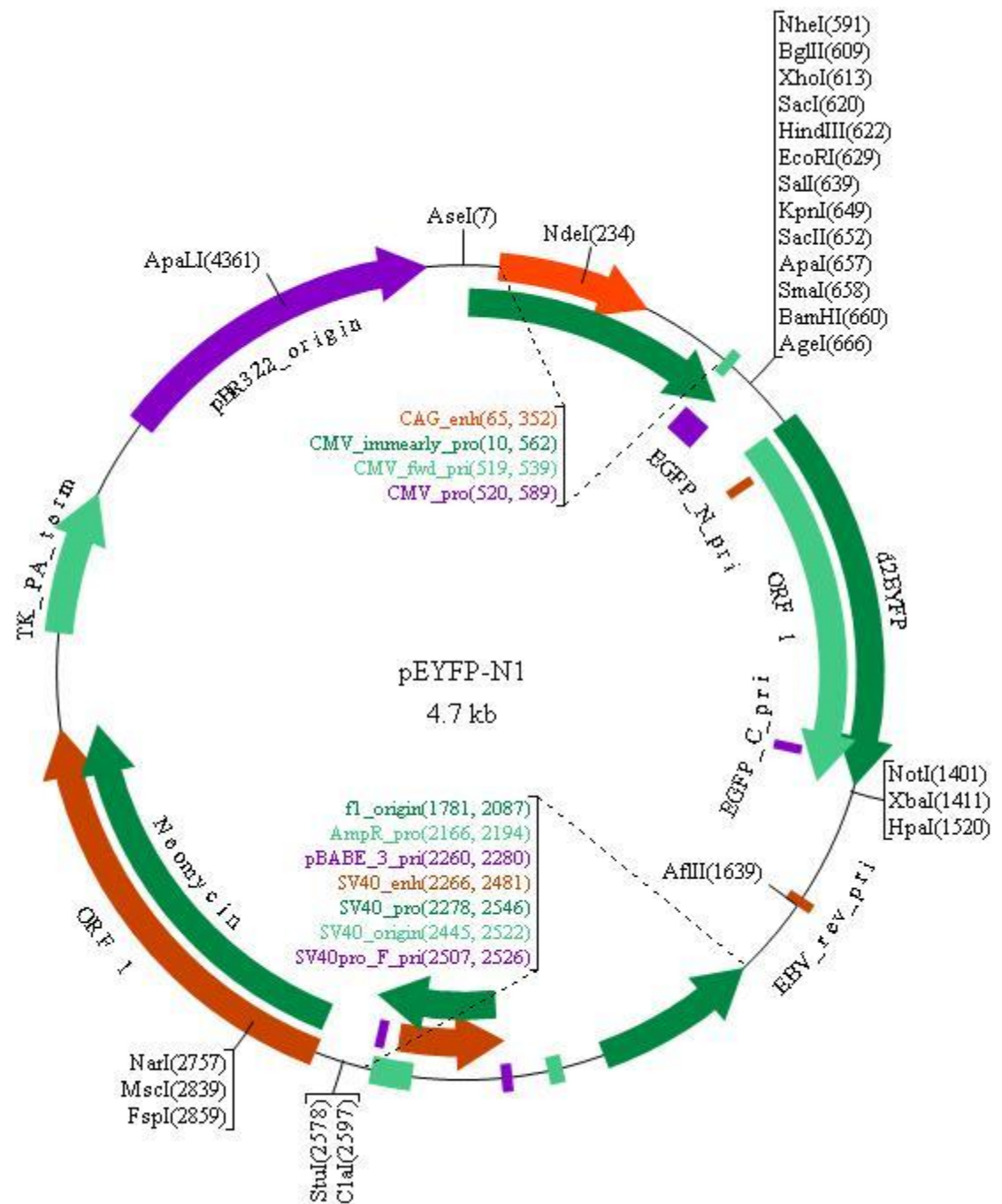
Insert	Vector	Tag	Source
TNF-R1	pBABE-puro	FLAG (N)	Dr. Martin S. Kluger Yale University School of Medicine
TNF-R1 c. 201_202del	pBABE-puro	FLAG (N)	Refer to pg.
TNF-R1 xx000/000xxx	pBABE-puro	FLAG (N)	Refer to pg.
TNF-R1 W239A	pBABE-puro	FLAG (N)	Refer to pg.
IL-1R1	pcDNA3	--	Dr. Vishva Dixit Dept of Physiological Chemistry Genentecg Inc. South San Francisco, CA USA
IL-1R1 K357/360/378R IL-1R1 K527R IL-1R1 K527/532R IL-1R1 K445/446R IL-1R1 K548R	pcDNA3	--	Refer to pg.
TLR-4		YFP (C) YFP (N)	Dr. Doug Golenbock Addgene 13018 Dr. Terjerk Espevik Norwegian University of Sceince and Technology
1 aa- TLR-4	pcDNA3	HA (N) FLAG (C)	Refer to pg.
TLR-4 Y655A TLR-4 Y674A TLR-4 Y680A	pcDNA3	YFP (C)	Refer to pg.
PS1 PS1 D257/385A PS2 PS2	pcDNA3	FLAG FLAG	Dr. Vishva Dixi Dept of Physiological Chemistry, Genentech Inc. South San Francisco, CA USA
TRAF6 TRAF6 DN TRAF6 C70A TRAF6 K124R	pRK5	HA HA HA HA	Dr. Ruaidhri Carmody Institute of Infection Immunity and Inflammation University of Glasgow

Insert	Vector	Tag	Source
Ubiquitin Ubiquitin K48R Ubiquitin K63R Ubiquitin K ϕ	pcDNA3	HA	Dr. Ruaidhri Carmody Institute of Infection Immunity and Inflammation University of Glasgow
Ubiquitin K48 only Ubiquitin K63 only	pcDNA3	HA	Dr. Mathijis Baens Center for Human Genetics University of Leuven, Leuven Belgium
Dynamin 1aa WT Dynamin 1aa K44A	pEGFP-N1	GFP (C)	Dr. Pietro De Camilli Addgene 22163 Addgene 22197
RAB5 WT RAB5 DN (S34N) RAB5 DA (Q79L)	pEGFP-N1	GFP (C)	Dr. Aymelt Itzen, Max Planck Institute of Molecular Physiology Dortmund
RAB7 WT RAB7 DN (T22N) RAB7 DA (Q67L)	pEGFP-N1	GFP (C)	Dr. Cecilia Bucci Università del Salento Italy

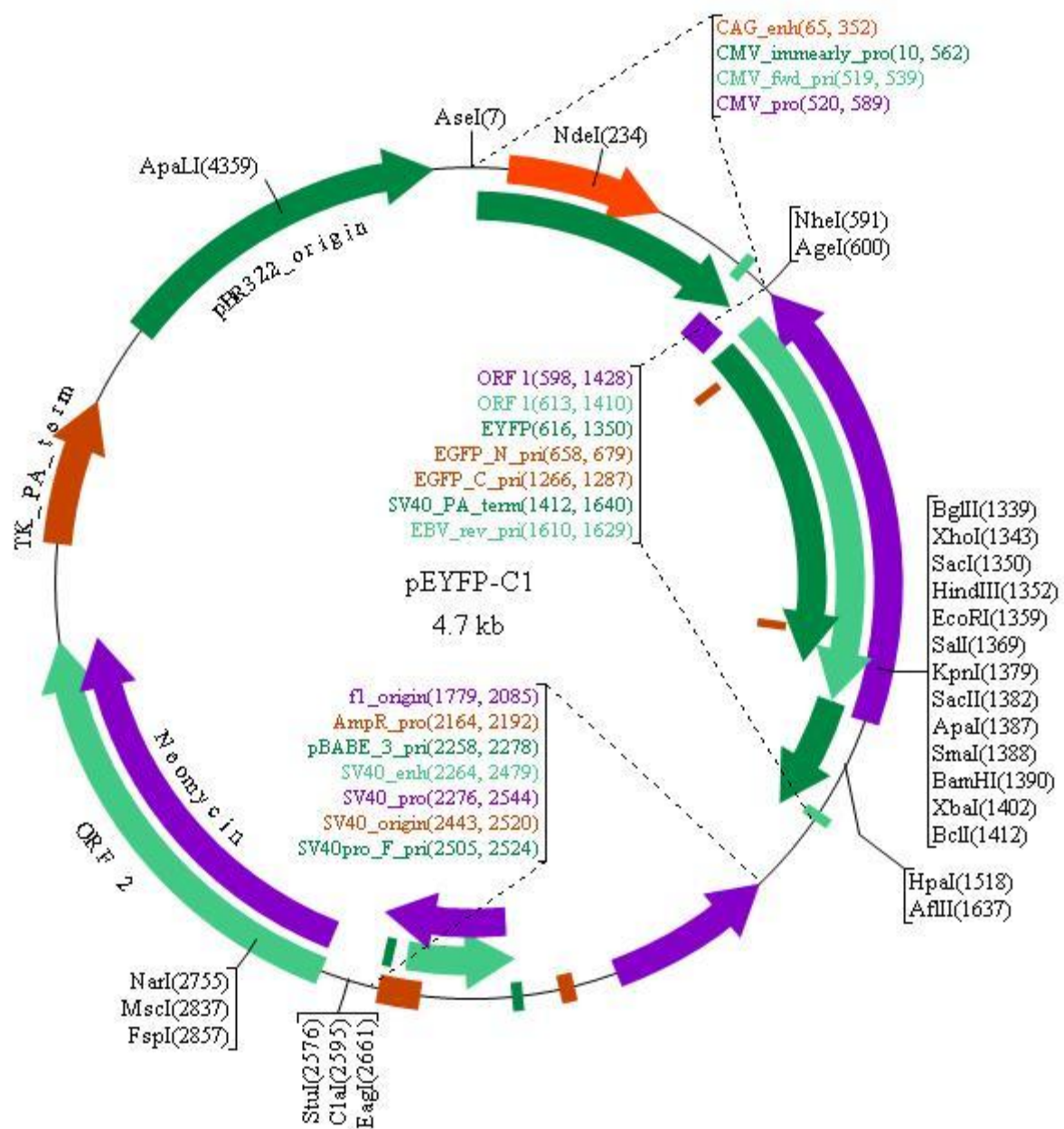
Appendix Table 3 Vector maps



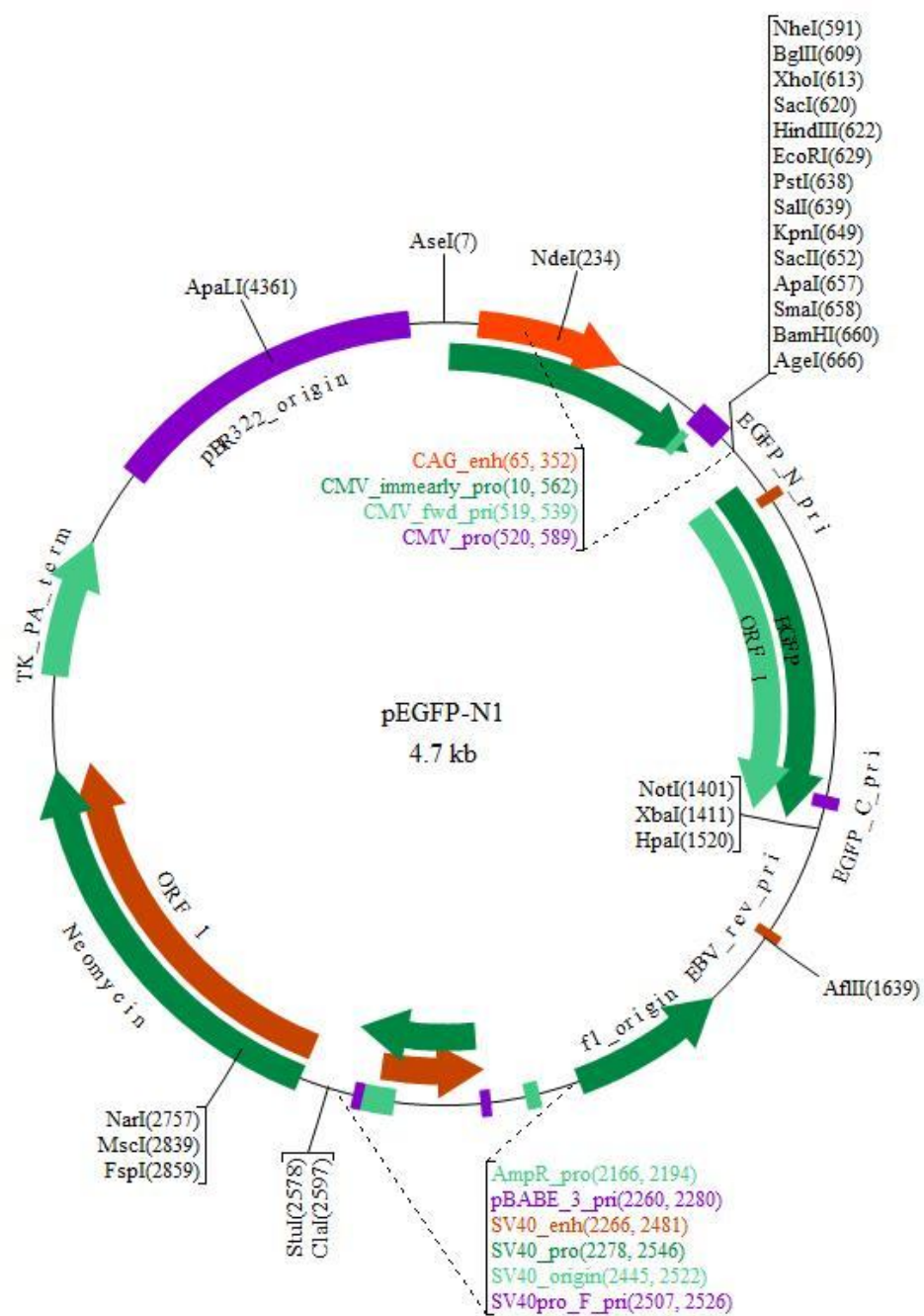
Appendix Table 3.1 Vector Map for pcDNA3 Source-Addgene



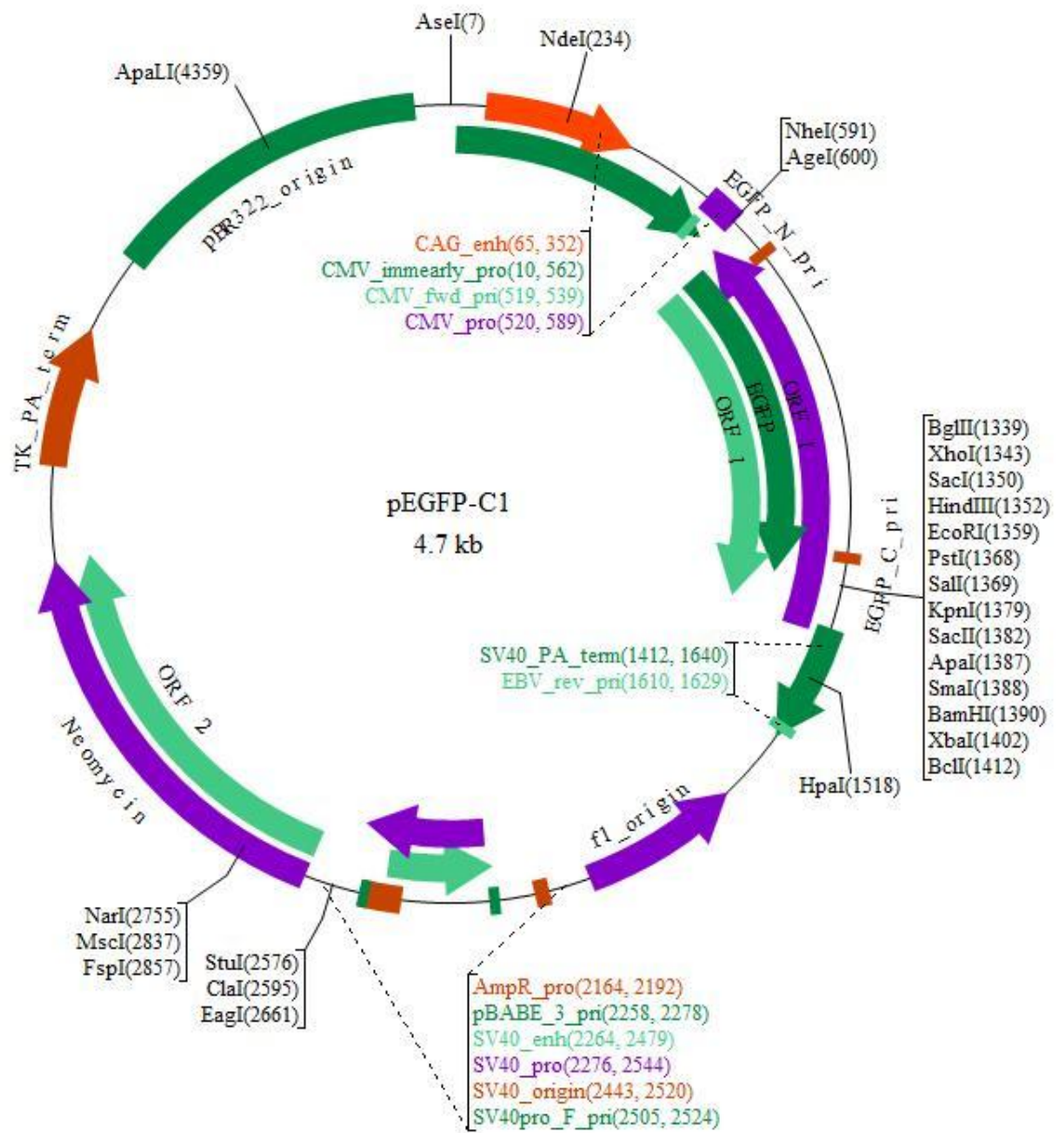
Appendix Table 3.2 Vector Map for pEYFP-N1 Source- Addgene



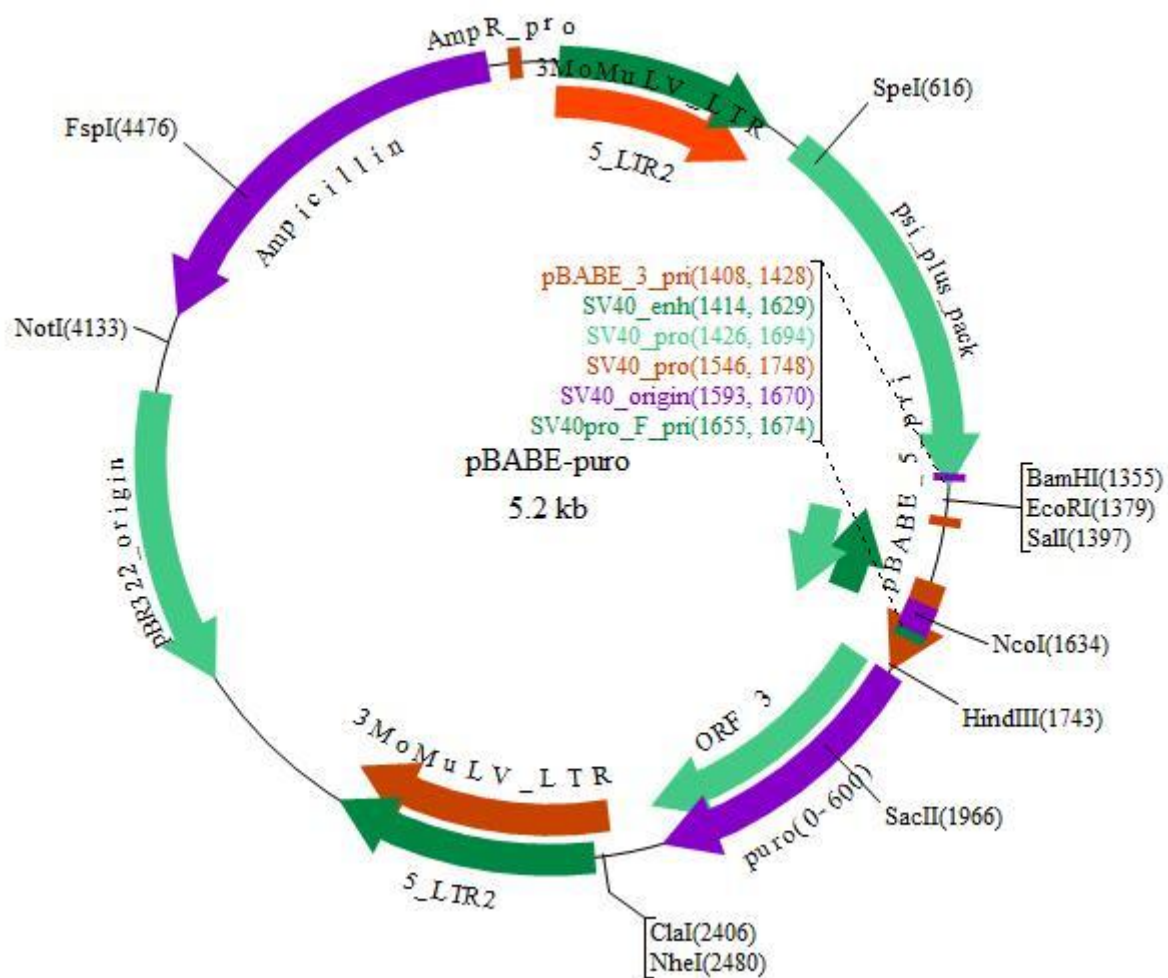
Appendix Table 3.3 Vector Map for pEYFP-C1 Source- Addgene



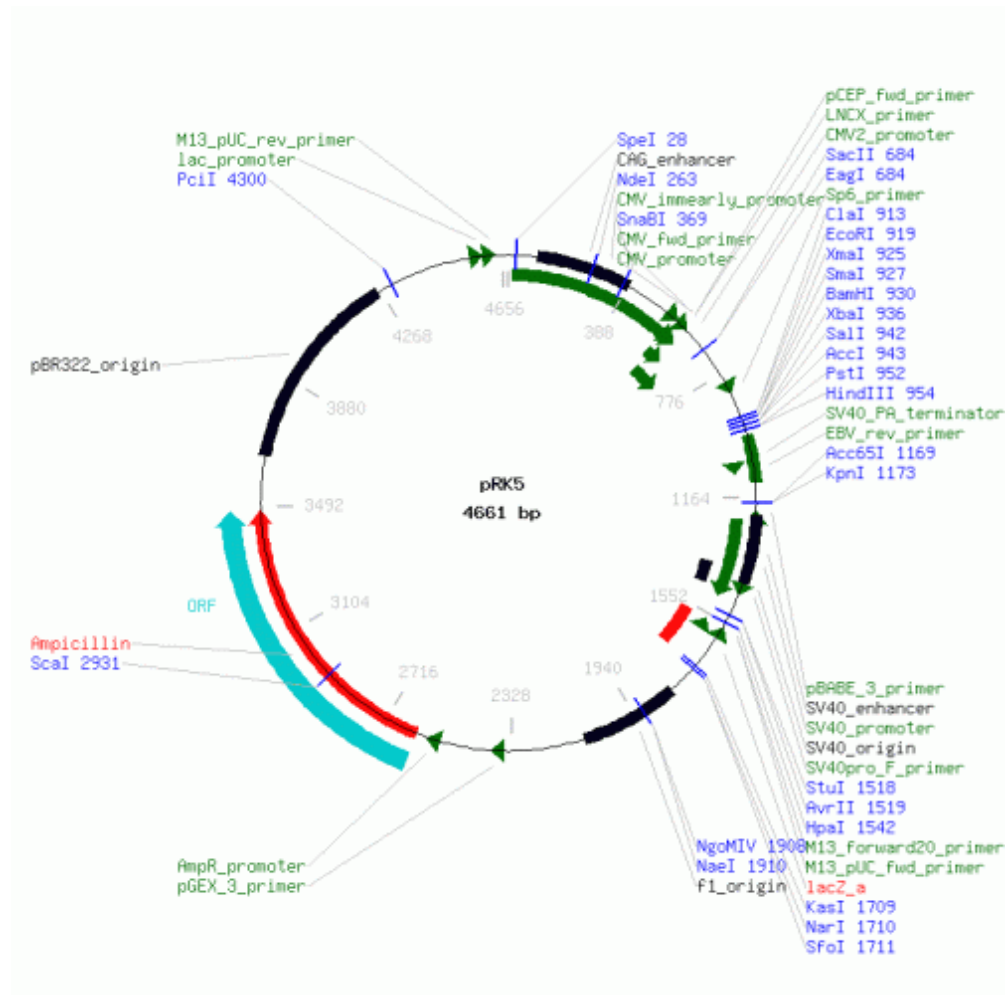
Appendix Table 3.4 Vector Map for pEGFP-N1 Source- Addgene



Appendix Table 3.5 Vector Map for pEGFP-C1 Source- Addgene



Appendix Table 3.6 Vector Map for pBABE-puro Source- Addgene



Appendix Table 3.7 Vector Map for pRK5 Source- Addgene

Appendix Table 4 Antibodies used, their sources and catalogue number

Insert	Vector	Tag	Source
TNF-R1	pBABE-puro	FLAG (N)	Dr. Martin S. Kluger Yale University School of Medicine
TNF-R1 W239A	pBABE-puro	FLAG (N)	Refer to material and methods
IL-1R1	pcDNA3	--	Dr. Vishva Dixit Dept of Physiological Chemistry Genentecg Inc. South San Francisco, CA USA
IL-1R1 K357/360/378R IL-1R1 K527R IL-1R1 K527/532R IL-1R1 K445/446R IL-1R1 K548R	pcDNA3	--	Refer to material and methods
TLR-4		YFP (C) YFP (N)	Dr. Doug Golenbock Addgene 13018 Dr. Terjerk Espevik Norwegian University of Sceince and Technology
PS1 PS1 D257/385A PS2 PS2	pcDNA3	FLAG FLAG	Dr. Vishva Dixi Dept of Physiological Chemistry, Genentech Inc. South San Francisco, CA USA
TRAF6 TRAF6 DN TRAF6 C70A TRAF6 K124R	pRK5	HA HA HA HA	Dr. Ruaidhri Carmody Institute of Infection Immunity and Inflammation University of Glasgow

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